UNIVERSITY *of* TASMANIA

Pharmacological Modulation of Mood, Behaviour and Cognition

by

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Declaration of Originality

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List of Abbreviations

Acronym	Definition
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
Aβ	amyloid β-peptide
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
AVP	vasopressin
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBT	cognitive behavioural therapy
CR	conditioned response
CRF	adrenocorticotrophic releasing factor
CMC	carboxymethylcellulose
CMS	chronic mild stress
COMTs	catechol-O-methyltransferase
CoQ10	Coenzyme Q10
CS	conditioned stimulus
CSD	chronic social defeat
CSI	chronic social isolation
DA	dopamine
DCF	2', 7' – dichlorofluorescin
DG	dentate gyrus
DGC	dentate granule cells
DI	discrimination index
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide
DMT	di-methyl tyrosine
DOP	delta-opioid receptor
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
ES	escapable shocks
FA	Friedreich's Ataxia
FBS	fetal bovine serum
FC	fear conditioning
FE	fear extinction
FST	forced swimming test
GDP	guanosine diphosphate
GR	glucocorticoid receptor
GSH	glutathione

GTP	guanosine-5'-triphosphate
HPA	hypothalamic-pituitary-adrenocortical axis
HPC	hippocampus
IASP	International Association for the Study of Pain
IS	inescapable shock
KOP	kappa-opioid receptor
LD	light and dark model
LH	learned helplessness
LHON	Leber’s Hereditary Optic neuropathy
LTP	long-term potentiation
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MAT	perisynaptical monoamine transporters
MCS	the multiple conditioning system
MDD	major depressive disorder
MOP	mu-opioid receptor
mtDNA	mitochondrial DNA
NADH	reduced nicotinamide adenine dinucleotide
NE	norepinephrine
NOR	novel object recognition
NMDA	N-methyl-d-aspartate
OF	open field model
PBS	phosphate buffered saline
PD	Parkinson’s disease
PDYN	prodynorphin
PENK	proenkephalin
POMC	proopiomelanocortin
PFA	paraformaldehyde
PTSD	post-traumatic stress disorder
ROI	region of interest
ROS	reactive oxygen species
SGZ	subgranular zone
SRIs	serotonin reuptake inhibitors
SNRIs	selective noradrenaline reuptake inhibitors
SSRIs	selective serotonin reuptake inhibitors
SVZ	subventricular zone
TCAs	tricyclic antidepressants
TRD	Treatment resistant depression
TST	tail suspension test

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Abstract

Chronic stress can lead to major depressive disorder (MDD) as well as depression-related comorbidities, such as anxiety and memory loss. Frequent treatment failure of the currently used conventional monoamine-based antidepressants indicate the urgent need to develop novel antidepressants that act by a different mechanism. There is good evidence that pain and depression create a vicious cycle. While pain worsens the symptoms of depression, the resulting depression worsens the feelings of pain. Opioids are clinically used as the most important treatment against severe pain. With the identification of the three classic opioid receptors, (mu-opioid receptor (MOP), delta-opioid receptor (DOP) and kappa-opioids receptor (KOP)), an increasing number of novel ligands have been described that not only show opioid receptor-dependent analgesic but also antidepressant-like effects in pre-clinical models. These antidepressant-like effects of opioid receptor ligands are thought to be a consequence of their ability to modulate cellular stress responses as well as their effects on learning, memory and behaviour.

On the other hand, it was proposed that oxidative stress plays a relevant role in the pathogenic mechanisms underlying MDD. Reactive oxygen and nitrogen species modulate levels and activity of multiple neurotransmitters that are involved in the neurobiology of depression. Clinical studies in MDD patients reported reduced levels of endogenous antioxidants, impaired mitochondrial function and a disturbed redox equilibrium. However, several studies reported opioid-induced mitochondrial dysfunction by a mechanism that involves the generation of reactive oxygen species (ROS). In line with this, morphine has also been described to increase oxidative stress rather than to reduce it. Thus, the connection between opioids, depression and oxidative stress appears significantly more complex.

At present, antidepressant-like effects of both clinically used and experimental MOP or DOP agonists have been described in pre-clinical and clinical studies. We therefore hypothesized

that opioids that simultaneously activate the MOP and DOP receptor, such as the UTAS-derived experimental bi-functional opioid 1003 (unpublished data), could also produce antidepressant-like effects. To test this hypothesis, we first assessed the behavioural effects of morphine (MOP agonist) in models of depression (learned helplessness model, LH), anxiety (light and dark model, LD), locomotion (open field model, OF) and pain (hot plate (HP) and tail flick (TF) tests). Parallel to previous studies, morphine showed promising antidepressant-like effects that were similar to the clinically used tricyclic antidepressant imipramine. However, morphine also enhanced avoidance learning and produced significantly higher physical activity after repeated administrations. This raises the possibility that morphine leads to behavioural changes that only mimic an antidepressant-like effect in this specific model. Therefore, the interactions between locomotion, learning and the antidepressant-like effects of drugs in the LH model require careful analysis and interpretation of multiple parameters. In this model, compared to morphine, the same dose of our novel bi-functional opioid receptor ligands (1001 and 1003) failed to produce any behavioural effects. Nevertheless, 1001 and 1003 somewhat reduced the oxidative damage in the dentate gyrus (DG) of both anterior and posterior hippocampus (HPC), an area of the brain that is highly involved in the regulation of mood, learning and memory.

Therefore, to understand the relevance of oxidative stress in MDD, a clinically used and much more potent antioxidant was assessed in our animal model. In rats that were exposed to repeated inescapable foot shocks in the LH model, antioxidant treatment significantly reduced oxidative cell damage in the DG of the anterior HPC and at the same time fully reversed the cognitive impairment associated with this model. However, this antioxidant treatment did not have any effects on the depressive-like symptoms in this model, nor did it modulate pain perception or anxiety levels.

Overall, these results highlight that opioids could be developed as antidepressants but also emphasize the need to investigate their off target effects in much more detail to judge the relevance of pre-clinical models before the start of clinical trials. The results of this study strongly suggest that oxidative stress in the HPC is a consequence of depression rather than a causative pathology. On the other hand, our results indicate that depression-induced cognitive deficiencies are a consequence of oxidative stress as antioxidant treatment was able to normalize this pathology. In addition to the evaluation of two novel bifunctional opioids for their potential use as anti-depressants, this project has provided new data towards the close relationship between pain, depression, cognition, and oxidative stress.

Chapter 1 Overview

1.1 Stress-based mental disorders

1.1.1 Depression

Major depressive disorder, also known as clinical depression, is a serious mood disorder with a lifetime prevalence of 13 % in men and 24 % in women in developed countries (1). In 2007, a study from the World Health Organization (WHO) estimated that depression affected health more profoundly compared to many other chronic diseases (2). As depression is often comorbid with other health conditions, there is an urgency to both improve its treatment and reduce its burden. Although clinical symptoms of depression vary, patients generally struggle to cope with their daily personal and social lives. They experience a loss of self-worth, disturbed sleep, reduced pleasure, reduced ability to concentrate as well as increased fatigue and irritability (3). At its worst, depression is an important risk factor of suicide (4). In 2012 alone, depression caused a million deaths worldwide and contributed to 12.5 % of all suicide cases caused by mental disorders (5,6) This represents a serious but unmet public health problem.

1.1.1.1 Physiological mechanisms of depression

The complexity of depression is reflected by the variety of known causal factors including genetic/epigenetic susceptibilities, environmental factors and medications. In addition, depression can also occur as a secondary pathology of other disorders. Although genetic factors are thought to be responsible for up to 50 % of depression cases (7), recent advances in the epigenetics of depression suggest that regulation of specific genes but not actual sequence variations in the form of polymorphisms may contribute to the highly heritable nature of depression (8,9).

It is widely known that chronic stress is associated with the onset of depression (10,11,12). Significant evidence suggests that most of the depression cases are likely a consequence of prolonged exposure to stressful conditions throughout life (13,14,15,16). This is well

established that chronic or lifetime stress can disturb adaptive systems of organisms, which lead to pathophysiological changes in brain function and structure as well as the development of depressive symptoms (17). The stress-induced adaptive system highly relies on the function of the hypothalamic-pituitary-adrenocortical (HPA) axis. Briefly, adrenocorticotrophic releasing factor (CRF) and vasopressin (AVP) are secreted from the hypothalamus upon initial stress exposure, which in turn activate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland to stimulate the secretion of cortisol (human) or corticosterone (rodents) from the adrenal cortex. The release of glucocorticoids changes energy metabolism, immune and inflammatory reactions that rapidly adapt the brain and body (18). The glucocorticoid receptors (GR) in the HPA axis and the limbic system are activated by glucocorticoids (19). Subsequently, the limbic system is altered to remember the stressful experiences and the corresponding stress responses are recorded for future events.

However, prolonged stress exposure leads to dysregulation of this stress-induced adaptive system and decreases its efficiency to cope with upcoming events, which ultimately will result in the development of depression (20). As a consequence, an overstimulated HPA axis and the increased levels of glucocorticoids have been frequently reported in patients with depression (21,22,23,24,25). In healthy subjects, plasma cortisol concentration of 8.6 ng/dl were detected, while MDD patients showed 12.9 ng/dl (26), similar to additional clinical studies (27,28).

To substantiate this connection in a preclinical model, rats were treated with similar concentrations of corticosterone (29). Over 3 weeks, daily injection of 40 mg/kg corticosterone produced plasma corticosterone levels of 568 ng/dl that were associated with depressive-like symptoms (30). In another rat study only 1 mg/kg corticosterone (s.c.) for 4 days generated similar plasma corticosterone concentrations of 590 ng/dl. However, despite similar plasma levels, this dose did not result in depressive-like symptoms (31). This discrepancy highlights that possibly corticosterone administration in pre-clinical models is only of limited use to

mimic cortisol dependent effects in patients. Based on the available information from animal studies, it is unclear if increased levels of glucocorticoids are causative to the development of depression as previously suggested (32), or merely represent a side phenomenon of depression. Brain-derived neurotrophic factor (BDNF) is a key protein that regulates the stress response (33) and affects the process of learning and memory formation (33). BDNF acts as a survival factor of developing hippocampal neurons, as well as affecting the morphology and viability of cultured adult dentate gyrus (DG) neurons (34). In animal studies, the expression of BDNF mRNA and protein was suppressed by corticosterone (35). In rats, a single dose of 1 mg/kg corticosterone (given an average weight of 250 g in rats, similar to a human equivalent dose of 0.16 mg/kg based on a bodyweight of 70 kg in humans (29)) reduced BDNF mRNA expression in the hippocampal DG by 70 % and suppressed BDNF protein levels in the same brain region by 17 % (36). Although supportive of a direct effect of corticosteroids on BDNF levels, a clear dose-effect for this response not available is so far, since a 10-times higher dose of corticosterone (single dose of 10 mg in rats with an average weight of 300 g (approximately 33 mg/kg, similar to a human equivalent dose of 5.38 mg/kg (29) based on 70 kg bodyweight) reduced mRNA expression of BDNF in the rat DG at a similar rate (37). A meta-analysis of studies published by July 2004 suggested that a single median dose of 25 mg cortisone (approximately 0.35 mg/kg based on a 70 kg person and equivalent to 2.17 mg/kg in a rat of 300 g) induced memory impairment in humans (38). In parallel, memory deficits were reported in rats that received corticosterone injections at the same equivalent dose (a single dose of 2 mg/kg in rats, equivalent to 0.32 mg/kg in humans) (39). The link between cortisone-induced memory loss and reduced BDNF mRNA and/or protein expression levels in the human brain is obviously not easy to demonstrate. Nevertheless, reduced mRNA expression of BDNF was detected in saliva in patients with first-episode psychosis, a mental disorder that is highly associated with psychosocial stress exposure (40). Those patients also showed reduced

cognitive function, when compared to healthy individuals (40), which supports the idea that BDNF has a regulatory role in stress circuits and stress-induced cognitive impairment. Another possible mechanism of depression-associated cognitive dysfunction is related to the capacity of the HPC for neuroplasticity, which is modeled by long-term potentiation (LTP) (41). Exposure to sufficiently severe stress, such as during the IS in the LH paradigm, can impair LTP in the rodent hippocampal DG (42), which was associated with stress-induced memory impairment in those animals (42,43). Despite gaps in our detailed understanding around dose effects and causality, the preclinical and clinical data both support the overall hypothesis that stress negatively affects synaptic plasticity and memory function.

Because of this established hypothetical link between stress and depression and depression-related cognitive deficits, this relationship has been used as the cornerstone for creating animal models of depression, which are vital for the study of this disorder as well as to identify and develop novel antidepressants.

1.1.1.2 Monoamine hypothesis of depression

The release of monoamine transmitters from synaptic vesicles is essential for synaptic transmission (44). The enzymes that are needed for neurotransmitter synthesis are initially produced in the neuronal cell body and are then transported down the axon to the synapse cytoplasm. In the presynaptic nerve terminal, neurotransmitters are synthesized from precursor molecules (mainly aromatic amino acids such as tyrosine, tryptophan, phenylalanine) (45) and stored in synaptic vesicles (46). In response to action potentials, calcium ions from the extracellular fluid enter into the presynaptic cytoplasm. This increase in cytoplasmic calcium causes the synaptic storage vesicles to fuse with the presynaptic membrane to release neurotransmitters into the synaptic cleft which propagates the neurotransmission process (46) (**Figure 1**). The termination of this process takes place by different mechanisms. The primary

one is the reuptake of excess neurotransmitters into the presynaptic neuron by perisynaptical monoamine transporters (MATs) which act as Na^+/Cl^- dependent co-transporters. Plasma-membrane localized Na^+/K^+ ATPases generate the required ion gradient to drive this process. Subsequently, the cytoplasmic neurotransmitters are either incorporated into synaptic storage vesicles via the vesicular amine transporter system (47) or are oxidized by monoamine oxidases (MAO) that are present on the surface of presynaptic mitochondria (38) (**Figure 1**). Any remaining monoamines in the synaptic cleft can be taken up by the post-synaptic neurons, where they are inactivated by the catechol-O-methyltransferase (COMT) by methylation (48). In the 1950s, researchers started to pay attention to the link between depression and monoamine deficiency and several lines of evidence have provided support for this hypothesis (49,50). Reserpine, which is an antihypertensive drug that depletes the monoamine neurotransmitters from peripheral sympathetic nerve endings, induced depression in 15 % of patients at higher doses (51). This finding suggested that agents that inhibit the depletion or breakdown by monoamines in the brain could also ease symptoms of depression. In addition,5-HT(1A) receptor agonists that increase serotonin (5-HT) signaling, increased the mood of patients with unipolar depression, likely by a post-synaptic mechanism (52,53)Together, these observations imply that abnormally low monoamine levels in the brain can cause depression. In the 1960s, researchers detected relatively low synaptic levels of norepinephrine (NE) receptors in some brain circuits, such as the hypothalamus, locus coeruleus and cortex, in depressed individuals (54), which suggested that depression could be treated by up-regulating expression of post-synaptic NE receptor (55). From the 1990s, research into the relationship between 5-HT and depression attracted most of the scientific attention. The distribution of a large majority of cell bodies of serotoninergic neurons is restricted to the brain stem of human brain. They are predominantly found along the midline of the raphe nuclei (56,57), where they are implicated in mood regulation (58). These serotoninergic neurons project from the raphe nuclei to many

forebrain structures that participate in the regulation of different functional systems such as the motor, somatosensory and limbic systems (59). They have also been associated with a wide range of neuropsychiatric and neurological disorders (60). Furthermore, the densities of both serotonergic neurons and postsynaptic receptors significantly decreased in patients with MDD, compared to healthy subjects (61,62). All of this evidence suggest that monoamine (e.g., 5-HT and NE) deficiency can lead to the development of depression (**Figure 1**).

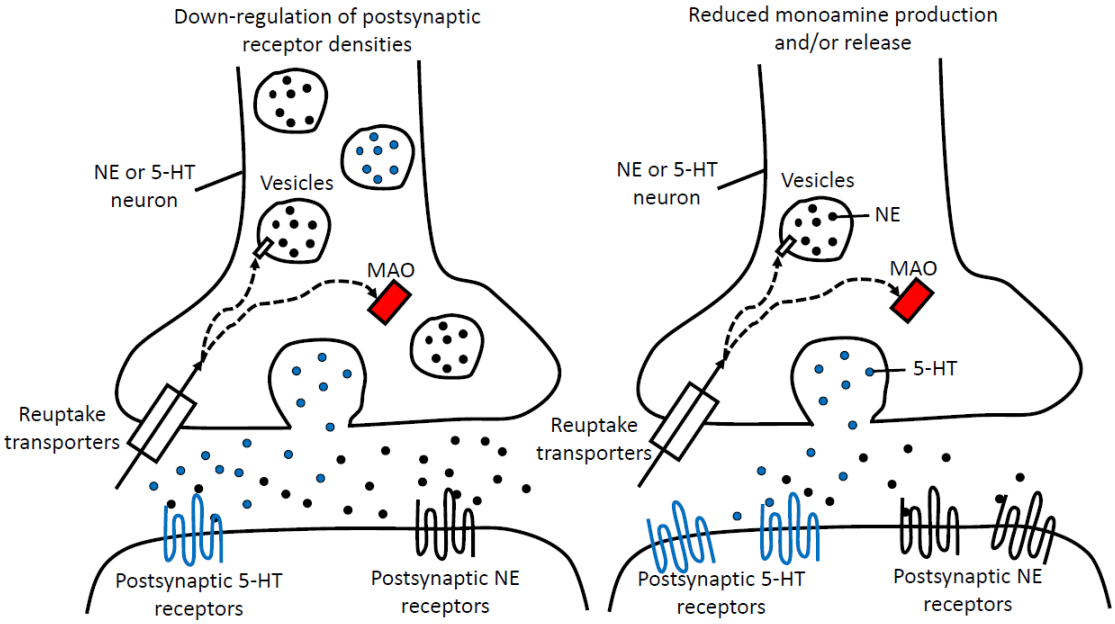

Figure 1. The monoamine theory of depression.
This theory states that reduced synaptic activities of monoamines (e.g., 5-HT and NE), are the consequence of either down-regulated postsynaptic receptor densities or monoamine depletion in pre-synaptic neurons. The core hypothesis suggests that the declined synaptic activities of monoamines can be regarded as the cause of depression.

This hypothesis is further supported by the therapeutic effects of monoamine oxidase inhibitors (MAOIs) in depressed patients. MAOIs have a long history of use in the treatment of depression (63). Commonly used irreversible MAOIs such as tranylcypromine, phenelzine and isocarboxazid provide a comparable response rate of 50 to 70 % in MDD patients, similar to that of tricyclic antidepressants (TCAs) (64,65). Approximately 50 % of treatment-resistant patients respond to MAOIs, which is significantly higher than some of the frequently described

TCAs, for example, imipramine (66). Therefore, MAOI are considered a better treatment option for treatment-resistant depression at later stages (66). In addition, the response rate of first generation MAOIs was also superior to placebo in depression with atypical features such as anxiety, fatigue and weight gain (67). Subsequently, second generation reversible MAOIs including selegiline, meclobemide and tyramine were developed to avoid the potential risk of hypertension that was associated with intake of specific food items such as cheese (68). Even though some of those reversible MAOIs, for example, moclobemide and selegiline, showed improved safety profiles while providing similar response rates compared to TCAs, serotonin reuptake inhibitors (SRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs), they do not replace the role of irreversible MAOIs for the treatment of atypical depression (63). Monoamine oxidase (MAO), as the main target of MAOIs, are localized to the outer mitochondrial membrane (69,70,71) and play an important role in the metabolism of catecholamines, such as dopamine (DA), NE and 5-HT (72). MAO break down catecholamines that are not protected by vesicles after re-uptake, which directly results in reduced levels of monoamine neurotransmitters in the neuronal synapses (73). Parallel to pre-clinical results, increased MAO activity and/or levels have also been reported in patients with depression (74). In addition, the metabolism of catecholamines by MAO is accompanied by the generation of hydrogen peroxide (H_2O_2), a cytotoxic agent that can lead to oxidative stress. Oxidative stress can in turn cause mitochondrial DNA (mtDNA) damage (75), lipid peroxidation (76), cellular damage (77) and eventually, mitochondrial dysfunction (78) (**Figure 2**). Recent studies suggested that the development of various stress-based disorders, including anxiety (79) and MDD (80), are highly associated with oxidative stress and mitochondrial dysfunction. This observation further supports the involvement of MAO in depression. It also implicates disturbed mitochondrial function in the pathology of affective disorders.

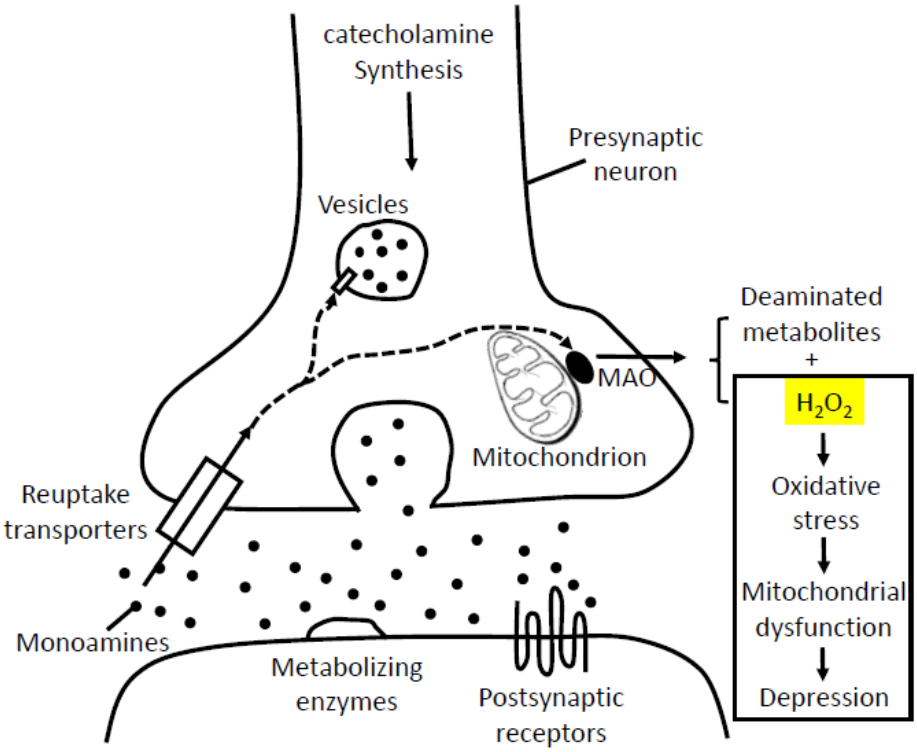

Figure 2. The role of monoamine oxidase in the development of depression.
Monoamine oxidase (MAO) metabolises catecholamines, leading to reduced levels of monoamine neurotransmitters. Hydrogen peroxide (H2O2) is generated during the MAO-mediated metabolism of catecholamines, which further result in oxidative-stress-induced mitochondrial dysfunction. Both reduced monoamine levels and impaired mitochondrial function could contribute to the development of depression.

1.1.1.3 Depression and cognition

Depression not only changes the way we feel, but also changes the way we perceive ourselves and the world around us. According to the current diagnostic criteria, uncontrollable recurrent negative thoughts are recognized as symptoms of depression (81). This type of cognitive disturbance in MDD has been considered as a key component of depression (82). In 1976, the cognitive theory of depression was proposed, which suggests that people's attitudes, thoughts, interpretations, and the way they recall stressful events can trigger the development and recurrence of depression (83). According to this theory, one of the most effective interventions for depression is to modify cognitive patterns through cognitive therapy, which can also be helpful to improve other symptoms of MDD such as sustained negative mood and lack of

interest (84). Indeed, cognitive impairment has been reported in patients with depression for over a decade (85). This phenomenon was supported by a study in elderly depressed patients that exhibited learning deficits (86). Later, more studies reported poor memory tests performance in patients with depression at all ages (87,88,89). In depressed patients with memory problems, reduced hippocampal volume was described (90). Because the HPC is one of the brain regions highly involved in regulating mood and cognitive function (91,92), such anatomical changes in the HPC could account for the observed cognitive deficits. . In addition, adult neurogenesis in the HPC may also be involved in the learning and memory process (93). Under laboratory conditions, many genetic and environmental factors that cause changes in cognitive performance affect hippocampal neurogenesis. Mutant mice with impaired performance on HPC-dependent learning tasks showed decreased neurogenesis in the subgranular zone (SGZ) of the hippocampal dentate gyrus (94,95). Similarly, poor spatial memory that was observed in the Morris water maze was associated with reduced SGZ neurogenesis across different mouse strains (95,96). In addition to the genetic factors, the environment has a big impact on SGZ neurogenesis. It was demonstrated that voluntary running increases SGZ cell proliferation and exposure to environmental enrichment promotes the survival of immature neurons (97,98). Both factors improve the performance of young and aged rodents in the spatial memory task (97,98). Furthermore, an enriched environment is able to enhance recognition memory (99). In contrast to physical exercise and environmental enrichment, aging and stress, conditions associated with cognitive deficits, negatively affect SGZ neurogenesis (100,101). Collectively, in the preclinical studies, adult neurogenesis in the HPC shows correlation with the learning and memory function. Similar to rodents, neurogenesis has also been described in adult human brains in the SGZ and the subventricular zone (SVZ) of the lateral ventricle (102,103,104). The association of adult neurogenesis with some physiological and pathological conditions such as epilepsy (105), stroke (106), AD (107)

causing cognitive dysfunction has now been investigated using postmortem tissues. In those studies, signs of migrated newborn neurons from SVZ to repair the sites of injury and increased cell proliferation in the SGZ in patients have been reported (108). Even though direct evidence for a link between adult neurogenesis and human cognition is not available yet, the changes to neurogenesis in postmortem specimens suggest a potential contribution of neurogenesis in the learning and memory function of adult humans.

1.1.2 Anxiety disorders

Anxiety disorders are widely observed in the general population and have been reported globally (109). According to an Australian national household survey in 2007, the prevalence of anxiety disorders over a 12-month period was 14.4 % out of 5300 survey responders (110). Anxiety disorders are associated with impaired workplace performance and significant economic costs (111). For example in Europe, the cost of anxiety disorders to society was estimated to be € 74.4 billion in 2010 alone (112). In addition, anxiety also increases the risk of cardiovascular morbidity and mortality (113). A meta-analysis of studies between 1980 and 2009 suggested that anxiety increases the incidence of cardiovascular disease in otherwise healthy individuals by 27 % (114). Fortunately, the diagnostic assessment and treatment options for morbid anxiety or anxiety-related disorders have improved considerably in the past three decades (115). For example, the use of psychotherapies such as prolonged exposure and cognitive processing therapy in America increased by 2 % every 5 years between 1995 to 2010 (116). In a 12-week study in the USA in 2008, 81 % of children/adolescent participants showed reduced PTSD symptoms when cognitive behavioral therapy was combined with a selective serotonin reuptake inhibitor (SSRI), compared to pharmacological intervention (55 %) or psychotherapy (60 %) alone (115). These data suggest that a combination of psychotherapies

and antidepressants treatment is a more effective treatment option against anxiety disorders than monotherapies.

1.1.2.1 Anxiety and depression

Anxiety disorders and depression disorders share many overlapping symptoms such as fatigue, impaired concentration, irritability and sleep disturbance (117). They may also share a common pathophysiology since exposure to stress is also one of the causes of anxiety disorders (118). The US National Comorbidity Survey reported that 58 % of patients with major depression fulfilled criteria for at least one anxiety disorder and most patients with primary anxiety disorders also experience major depressive episodes (119). Approximately 68 % of individuals with co-morbid depression and anxiety had a history of being anxious for at least a decade before the development of depression, which suggests common mechanisms between these two mental diseases (120). Anxiety and depression seem to have separate symptom clusters according to the results of traditional factor analysis from patients with symptoms of either depression or anxiety or both (121). The distinction between anxiety and depression is subject to an ongoing debate due to the high comorbidity between these two disorders (122). One line of thought argues that the two disorders manifest different symptoms along a spectrum of disorders (123), while others propose that anxiety and depression are clearly separate entities (124). At present, more research is needed into the pharmacological interventions, diagnostic criteria and pathopsychological mechanisms of both disorders, before this debate can be decided.

1.1.2.2 Posttraumatic stress disorder

One of the common seen anxiety disorders is PTSD, which occurs in a group of individuals exposed to traumatic events (125). It comprises four clusters of symptoms, including intrusive

symptoms, persistent avoidance of stimuli, changes in mood and cognition and marked adjustments to reactivity and arousal (126). Over the past 30 years, numerous studies investigated the biological, cognitive and behavioral characteristics of PTSD and have established reliable methods for diagnosis and some limited treatment options (127).

1.1.2.2.1 Epidemiology

According to the Australian National Mental Health and Wellbeing Survey in 2007, the prevalence of PTSD was estimated to be around 6.4 % (110). However, there is uncertainty towards the robustness of this survey, since a prevalence of 4.4 % was reported in a different study (128). In this study, the impact of comorbidities and sociodemographic factors on prevalence of anxiety disorders were also considered, apart from targeting the methods of data collection and analysis. Over a decade (2007 - 2017), the prevalence of PTSD in Australia increased by 3.2 % (129). The likelihood of contracting PTSD is largely depended on the nature of potentially traumatic events and the intensity of psychological trauma. Based on the report of the Australian Centre for Posttraumatic Mental Health in 2013v war trauma is the biggest risk factor for PTSD (130). According to several studies between 1988 and 2008, PTSD has been frequently reported in returning American veterans since the Vietnam War (131,132,133,134). However, there is also a significant amount of uncertainty regarding the reported numbers since the lowest prevalence (2.2%) of combat-related PTSD was reported in 1988 (131), while the highest prevalence (16.8%) was reported only two years later (132). Even though PTSD cases in Australian veterans are lower at present, similar incidences compared to US soldiers seem likely (135,136). The 12-month prevalence of PTSD in the general Australian population is also associated with other risk factors, such as a history of rape (9.2 %), sexual assault (5.5 %), witness of killing or death (0.6 %) and natural disasters (0.3 %) (137). In addition, the risk for developing PTSD also shows a high gender bias, with a 2 :

1 ratio for females over males (138) and there is a higher risk to develop PTSD after experiencing traumatic events at a younger age (139). Approximately 2 in 3 children experience a traumatic event by the age of 16 and 13.4 % of those will eventually develop some symptoms of PTSD (139).

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1.1.2.2.2 Classification

PTSD is caused by exposure of individuals to traumatic events that causes them to experience intense fear, horror, humiliation and/or grief (140). As defined by the Diagnostic and Statistical Manual of Mental Disorders, a traumatic event pertains to an experience of threatened or actual death, serious injury or sexual abuse that is either directly experienced or witnessed by the individual. However, the description of a traumatic event is very broad, allowing it to encompass a range of scenarios. Some examples of traumatic events include natural disasters, terror attacks, domestic and sexual violence, repetitive trauma exposure, death of a family member or of a close friend (141). In addition to the broad definition of what classifies as a traumatic event, the experience of each affected person is unique and highly depended on individual differences in processing and interpreting those events. Furthermore, in approximately 80 % of individuals with PTSD, their condition is complicated by additional psychiatric disorders, such as depression (142), substance abuse (143) and other anxiety disorders (144). It is also clear that depression could trigger the development of PTSD once the patient is exposed to a similar traumatic event (145). Those comorbidities likely affect the presentation and clinical course of PTSD, which is why there is no strict clinical descriptions towards the complex clinical presentations of PTSD. This uncertainty largely contributes to the difficulty to establish accurate diagnostic criteria and a large degree of PTSD misdiagnosis cases due to the associated comorbidities (146).

1.1.2.2.3 Clinical presentations and diagnostic criteria

For a PTSD diagnosis to be considered, it is necessary for a person to exhibit at least one symptom out of each symptom cluster (i.e., intrusive symptoms, persistent avoidance of stimuli, changes in mood and cognitions and marked adjustments to reactivity and arousal). At present, there are four diagnostic criteria used to identify PTSD symptoms. The first criterion, the manifestation of intrusive symptoms, is the most identifiable symptom of PTSD, which is highly recognised by the public. Intrusive symptoms may present as flashbacks or nightmares among other manifestations in the presence of stimuli (140). These symptoms are often associated with intense, uncontrollable physical reactions that are similar to those supplementary to an anxiety attack (147).

The second criterion is the avoidance of stimuli and can be immensely disruptive to everyday life. Avoidance relates to the conscious decision to elude any reminder of the traumatic event, and the extent to which patients might go can often be quite extreme (148).

Thirdly, negative changes to cognition and mood covers a large range of signs. Changes to mood may present as feeling depressed and not being able to feel positive emotions or find pleasure in activities formerly enjoyed (147). Changes to cognition may involve symptoms such as not being able to remember details of an event or associated situations. This symptom cluster also involves feelings of blame (149).

The final criterion involves symptoms of arousal and reactivity. The person may exhibit aggressive behaviour or an increased response to agitation (140). All the presented symptoms may be seen as over-reactive response to small everyday issues. The persons ‘flight or fight’ response is permanently switched on and the individual is constantly looking out for danger. This can cause the individual to be over alert of their surroundings leading, which negatively affects their ability to concentrate (147).

1.1.2.2.4 PTSD and cognitive dysfunction

Many individuals that suffer from PTSD also show cognitive disturbances. More than 70 % of military veterans undergoing PTSD treatment exhibit poor performance in memory tests (150). However, the levels of cognitive dysfunction appear different from patient to patient, which can mask its relationship with PTSD. The appearance of memory deficits in PTSD could be either similar to those observed in other neurological disorders such as depression-induced memory loss, or similar to pure memory impairment observed in dementia (151). The identification of a cognitive component of PTSD highly relies on collected sample targets and sizes (152). Therefore, the association between cognitive impairment and PTSD is still unclear. Yet such association may lead to poorer processing of traumatic memories and thereby contribute to subsequent development of PTSD symptoms and also impair therapeutic interventions based on fear extinction (FE) learning (153).

1.1.3 Chronic Pain

According to the International Association for the Study of Pain (IASP), pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage” (154). It can be caused by a number of conditions and diseases such as cancer or neuropathies (155). This disease remains a primary global public health concern, which is reflected by the high numbers of documented patients receiving analgesic treatment (156,157). Pain is classified by the IASP into multiple classes (axis) based on the region of the body that is involved (e.g., lower back pain), affected organs (e.g., headache), aetiology (e.g., neuropathic pain) and the intensity and duration of pain (e.g., acute or chronic pain) (158). Among these, chronic pain, which is used to describe persistent or recurrent pain that lasts for over 3 months, can be further classified based on the severity, causes and functional impairment. For example, chronic pain can be classified into chronic primary and chronic

neuropathic pain. In addition both of those subgroups can be associated with emotional distress and disability (159). Therefore, to provide effective therapeutic solutions for chronic pain, a variety of physical, psychological and emotional factors need to be considered (160). Over the past decades, brain imaging studies provided evidence for the preferential involvement of the prefrontal cortex in chronic pain (161,162). The prefrontal cortex plays an important role in recognition memory (163), working memory (164) and memory formation (165), which indicates a cognitive element in chronic pain. For example, 72 out of 170 patients with chronic pain complained about poor concentration, forgetfulness and difficulty in problem-solving (166). However, most of the established animal models, which largely rely on evoking a reflex nociceptive response through inducing noxious stimuli, show little about the cognitive component of chronic pain (167). Therefore, it is important to establish more comprehensive preclinical models to reflex diverse symptoms of associated chronic pain conditions, in order to effectively develop new treatment.

In addition, a connection between untreated or undertreated chronic pain and the development of depressive symptoms is well-established (168). Depression has been reported as a concomitant condition in untreated chronic pain, although there is a wide range of incidences (10 – 90 %) among neuropathic pain patients (169). The close connection between depression and pain is illustrated by the fact that more than 75 % of patients with depression display symptoms of untreated pain, whereas patients that suffer from persistent pain are more likely to develop depression (170). It seems the association between chronic pain and depression lies with two neurotransmitters, i.e., 5-HT and NE. For example, reduction in presynaptic 5-HT release and a compensatory upregulation of postsynaptic 5-HT_2 receptors were observed in depressed patients (171), while reduced presynaptic 5-HT release in chronic pain patients was also detected just as in depressed patients (172). Apart from the comorbidity with depression, chronic pain also co-occurs with different anxiety disorders, such as generalised anxiety

disorder, panic disorder and social phobia (173). Approximately 45 % adult patients with persistent pain also experienced symptoms of anxiety according to the US National Health Interview Survey in 2010 (174).

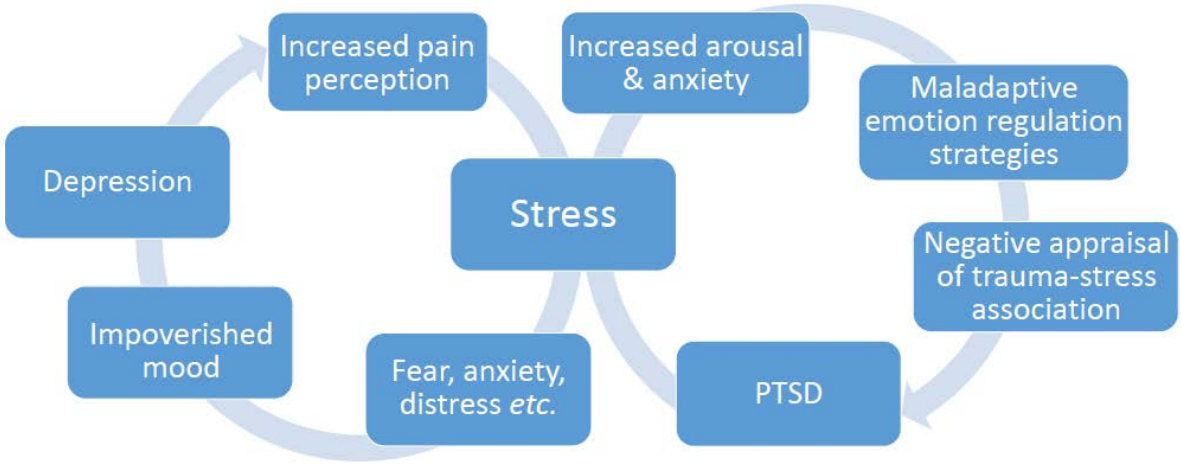

Figure 3. Vicious circles between stress, depression, anxiety and chronic pain.

Exposure to stress can cause mood disturbances and negative thoughts, which can lead to the development of anxiety and depression. In addition, increased pain perception that is observed in depressed patients, works as a stressor and further worsen the depressive symptoms. On the other hand, long-term maladaptive cognitive systems result in the development of posttraumatic stress disorder, which, in turn increases stress levels in patients.

1.2 Current treatment of stressed-based mental disorders

1.2.1 Depression

The molecular pathology of depression is still not well understood and current pharmacological treatments rely heavily on the monoamine theory of depression, which postulates that reduced brain neurotransmitter levels are linked to the manifestation of depressive symptoms (34). Current clinically used antidepressants are largely based on this theory and aim to increase levels of monoaminergic neurotransmitters in the synaptic cleft through inhibiting their re-

uptake or by reducing their metabolism, to ultimately increase the activity of the HPA axis (28,35). These drugs include SSRIs, SNRIs, MAOIs and TCAs. According to a meta-analysis until Jan 2016, which studied 21 clinically used antidepressants, all drugs showed positive treatment outcomes in adult MDD patients, compared to the placebo groups (175). Among those drugs, atypical antidepressants agomelatine, amitriptyline and venlafaxine, SSRIs escitlopram and paroxintine, TCA mirtazapine, and SNRI ortioxetine were the most effective interventions. However, compared to other classes of antidepressants, patients better tolerated SSRIs such as citalopram and escitalopram during the on average 8-week treatment periods (175). However, it seems different studies yielded inconsistent results in terms of side effects, efficacy and dropout rates of antidepressants. In another meta-analysis that looked at the data until July 2008, escitalopram (SSRI) provided overall more positive outcomes than placebo in the 22 trials, although showed no better efficacy and acceptability than other classes of antidepressants over the same average 8-week treatment period (176). The differences between studies could be due to sample size, the amount of retrieved information, diagnosis criteria and/or methods of data review (177). More importantly, those systemic reviews and meta-analysis arguably fail to provide reliable guidelines to current practice due to the lack of consistent results and standardised analysis protocols. Therefore, to overcome the drawbacks associated with conventional antidepressants, such as delayed onset and low response rate (178) and to provide reliable data for clinicians, improved study protocols to assess efficacy and acceptability of antidepressants are urgently needed.

A new generation of fast-acting antidepressants are under investigation that are developed based on neurotransmitters other than serotonin and noradrenaline with different neurological causes of depression(179). The N-methyl-d-aspartate (NMDA) receptor antagonist ketamine is one of the revolutionary antidepressant alternatives that showed efficacy in patients with treatment-resistant depression (TRD) (180). However, the majority of ketamine trials were

performed on small scales and variable efficacy has been reports across these studies. The initial trial was only conducted in 8 participants, where symptomatic relief was observed in depressed patients over the first 3 days after a single dose of ketamine (0.5 mg/kg) (181). Similar positive results were demonstrated at the same dose in 33 patients with treatment-resistant depression (182). In this study, a maximal efficacy of 2-weeks was reported in 2 patients (182). In a more recent trial, ketamine attenuated depressive symptoms over a one week period and provided higher response rates (64 %) compared to the 28 % response rate of midazolam (active placebo control) (183). Even though more clinical studies using chronic administrated ketamine (up to 19 days) have been conducted, the maximum duration of efficacy was only observed up to 28 days, demonstrating only a transient antidepressant profile of this fact acting drug (184,185). In addition, the efficacy of ketamine seems to be influenced by the administration methods of drug and the individual differences of patients. For example, both long-term sublingual (186) and i.v. infused ketamine (185) showed immediate effects for symptom relief, while this rapid therapeutical response was not observed using oral administration (184). Furthermore, patients with a history of alcohol dependence showed better therapeutical outcomes in response to ketamine, compared to MDD patients without alcohol abuse (187). The underlying mechanisms of the antidepressant effects of ketamine, as well as its variable efficacy in different trials are still not defined yet but more studies are underway to understand its molecular mode of action.

1.2.2 Posttraumatic stress disorder

Current therapeutic strategies for PTSD mainly consist of psychological counselling such as cognitive behavioural therapy (CBT) and pharmacotherapy. The implementation of CBT can decrease the severity of PTSD symptoms in comparison to a no-treatment group (188). Although psychological counselling is shown to be effective in some cases, there are significant

disadvantages. This form of therapy can be a lengthy process as well as an undoubtingly confronting experience to the patient. Active treatment approaches that are based on trauma-focused treatment as well as other methods of CBT have shown a high dropout rate (189) and a response rate as low as 50 % (190). The most promising therapeutic approach is proposed to be a combination of psychotherapy and pharmacotherapy, as this form of therapy is reportedly more effective for treatment of mental disorders than either mono-therapy alone (191).

SSRIs, which are commonly used for the treatment of depression, are the only class of drugs that are prescribed as a first-line therapy for PTSD in Australia (147). SSRIs and other conventional antidepressants are thought to only treat the symptoms of PTSD (147). Currently, paroxetine and sertraline are the only two drugs that are approved by the Food and Drug Administration for use in PTSD patients (192). However, it is common see a combination of SSRIs and SNRIs in clinical practice (193). The success rate of single medication treatment with an SSRI or SNRI was reported to be around 58 % (194). Although some improvements have been achieved by the administration of these drugs in PTSD patients (195), in many cases these drugs have shown no efficacy (196). For example, escitalopram (SSRI) failed to reduce the onset of PTSD at 9-month post-trauma exposure, compared to placebo control, in a multi-arm randomised controlled trial (197). A similar lack of evidence of efficacy was observed when using antidepressants imipramine and fluoxetine to reduce the symptoms of acute stress disorder in paediatric burn victims (198).

Several additional classes of medication may be useful in the prevention of PTSD. Treatments such as the β-adrenergic antagonists propranolol (199) and glucocorticoids such as hydrocortisone (200) also exhibited promising PTSD symptom relief in controlled trials. The result may support the idea of applying cortisol after trauma to prevent PTSD (201). However, conflicting results were seen in those classes of interventions. For example, a meta-analysis to February 2014 reported that propranolol showed no significant effect in reduction of PTSD

symptoms in total 345 patients compared to placebo (202). What's more, benzodiazepines such as clonazepam, temazepam and alprazolam are used in treating acute trauma (203). Nevertheless, the actual practice of using benzodiazepines in treating PTSD has been questioned by negative findings in a small randomised placebo-controlled trial (204). In this study, life-threatening incidents occurred after administration temazepam, leading to the development of PTSD in a large proportion of participants (204).

The currently accepted view for PTSD treatment is to use available drugs to create some relief for patients that undergo psychological treatment. However, conventional pharmacotherapy is unreliable and only provides symptomatic relief rather than curing the disease (196). It is important to note that the lack of well-validated and predictive preclinical models of PTSD significantly delays the development of effective treatment options. Therefore, an accurate and authenticated model, which assists in the understanding of the underlying mechanisms leading to PTSD, is a requirement for the development of novel pharmaco-therapeutic alternatives.

1.2.3 Chronic pain

1.2.3.1 History of the use of opiates and opioids

According to the Australian Medicines Handbook, opioids are strong analgesics and are used as first-line therapy for moderate-to-severe cancer pain and postsurgical pain (205). They are also recommended as second-line therapy for patients with neuropathic pain that do not respond to other non-opioid treatments (205). The use of opioid therapies in long term treatment of chronic pain is limited because of the initiation of analgesic tolerance (206). Opiates, derivations of natural poppy plant extracts, go by a variety of names including opiates, opioids, and narcotics. The term "opiates" is sometimes used for close relatives of opium such as codeine, morphine and heroin, while the term "opioids" includes also semi-synthetic drugs such as oxycodone (207) and fully-synthetic drugs such as methadone (208). The psychological

and physiological effects of opioids have been known for thousands of years. As early as 1550 BC, ancient Egyptians mentioned opium and described it as a drug of joy. Opium was also used to treat diarrhea, internal bleeding and pain at that time (209). In the 9th century B.C., Homer described the pain killing effects of opium in his work *The Odyssey* (210). However, from the 5th century B.C., the adverse effects of opioids were increasingly noticed and opioids were considered as toxic compounds, mainly due to their addictive potential and even lethal activity (211,212). In 1806, morphine was first isolated from opium (213) and was subsequently used as anesthetics in small surgeries following the invention of syringe and hollow needle (210). One and half century later, methadone and the first opioid antagonist nalorphine were synthesized (214). Nalorphine was the first bifunctional opioid that blocks the analgesic effect of morphine (MOP antagonism) but also acts as analgesics on its own due to KOP agonist activity (210,215,216,217). Even though nalorphine demonstrated morphine-equivalent analgesic effects in the clinic, its medical use is largely limited due to its KOP receptor-dependent psychological side effects such as dysphoria, anxiety, confusion, and hallucinations (218).

1.2.3.2 Endogenous opioid peptides

With the discovery of the enkephalins in 1975 (219), numerous larger opioid peptides, such as beta-endorphin, dynorphin 1 – 17 and peptide E were isolated, which contain the N-terminal sequence of either met-enkephalin or leu-enkephalin (220). Now it is clear that all opioid peptides derive from three different precursor proteins that include proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) (**Figure 4**) (221) and their precise structures have been identified using recombinant expression (222).

POMC is a glycopeptide that is glycosylated towards the N-terminal end of the molecule (223). Endogenous peptides derived from the POMC family are mainly produced in the pituitary

gland, from where they are released into the blood stream in response to stress and various endocrine signals (220). Biosynthetic experiments on isolated rat pituitary cells indicate that POMC is processed into beta-endorphin, which is further processed into beta-endorphin 1 – 27 in the rat intermediate pituitary (224). Beta-endorphin has been proposed to be an endogenous ligand for the MOP receptors because of its moderate MOP affinity (225). This peptide itself is an opiate-like peptide and has shown naloxone-sensitive antinociceptive activity after local administration due to its MOP agonistic activity (226). In addition, beta-endorphin also showed promising facilitating effects for the acquisition stage of memory formation in animal models (227). The second precursor proenkephalin (PENK) is cleaved into both Met-enkephalin and to a lesser extent, Leu-enkephalin, as well as into several intermediate opioid peptides such as peptide E, BAM-22P, -20P, -12P and peptide F (228). These peptides have been isolated and sequenced from bovine adrenal medullary tissues (229). Finally, prodynorphin (PDYN) is a common precursor of a series of opioid-like peptides such as Leu-enkephalins, α-neo-dynorphin, dynorphin 1 – 8 and dynorphin 1 – 17 (220). Both PENK - and PDYN - derived peptides have a wide range of distribution in the central and peripheral nervous systems, including the nucleus accumbens, the median forebrain bundle, the hypothalamus and the hippocampus of rat brain and the spinal cord, the adrenal medulla, the sympathetic ganglia and the gastrointestinal tract (230).

Enkephalins that are processed from either PENK or PDYN possess preferential affinity for the MOP and DOP receptors (231), while all other opioid-like peptides that are derived from PDYN, for example, dynorphin 1-8 and dynorphin 1-17, show a selective affinity for the KOP receptor (232,233). Beta-endorphin, the end product in the processing of POMC cleavage, has affinity for all three opioid receptor systems, but is specifically a highly potent agonist of the MOP receptor (234,235). Even though, none of the endogenous peptides are truly selective for only the MOP receptor, some of the intermediate PENK-derived peptides , such as peptide E and BAM-22P, exhibit a very high affinity to the MOP receptor compared to the other receptor types (236) and are able to induce substantial MOP-dependent analgesia after intracerebroventricular injection in rodents (228).

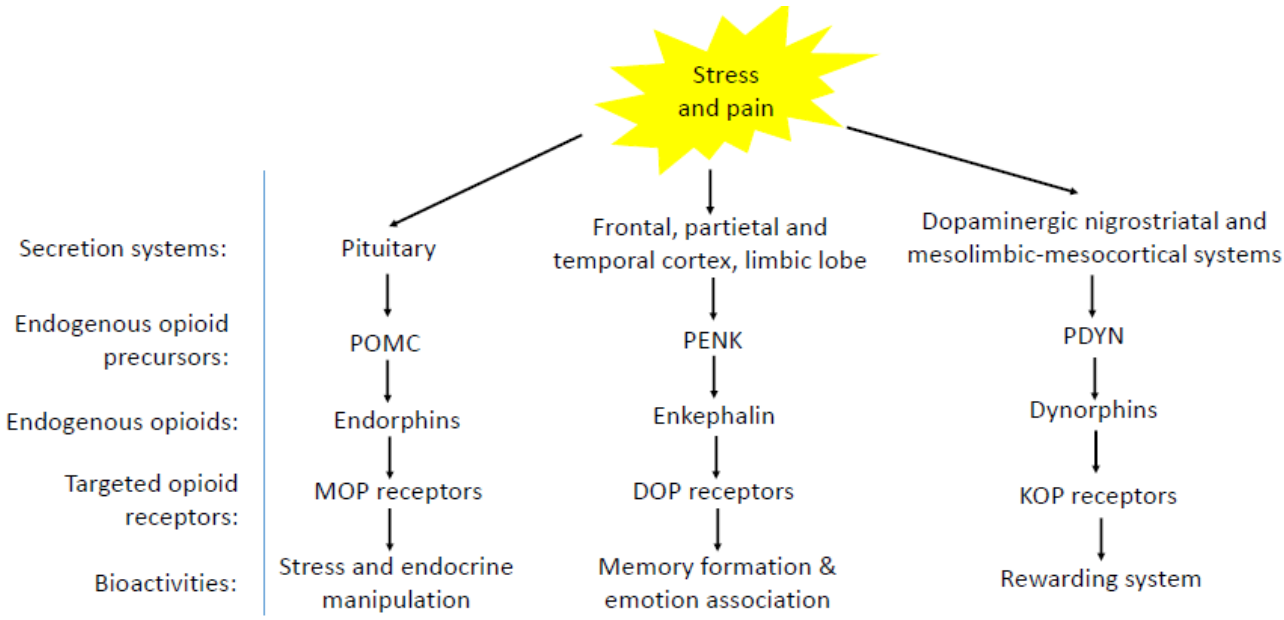

Figure 4. Endogenous opioids and their related precursors, opioid receptors and biological effects.
Precursors of endogenous opioids include proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN). They are secreted after exposure to stress and/or pain. These precursors are cleaved into endogenous opioids, which induce different biological activities by activating different opioid receptor types.

1.2.3.3 Classification and anatomy of opioid receptors

In the 1990s, the existence of three distinct types of opioid receptors was confirmed by molecular cloning long after the identification of endogenous opioids in 1975 (231). Opioid

receptors belong to the inhibitory G_i linked G-protein coupled receptor subfamily (237). G proteins consist of three protein subunits, including α, β, γ subunits. In the inactive state the α, β and γ subunits form a heterotrimeric complex. In this complex, the α subunit binds guanosine diphosphate (GDP) (238). After an opioid agonists bind to their specific opioid receptors, GDP is released from the α-subunit of G_i protein and is replaced by guanosine-5'-triphosphate (GTP). The binding of GTP activates the α-subunit, leading to its disassociation from the βγ-complex. Subsequently, this G_i-α protein inhibits adenylyl cyclases, which prevents the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (239). This mechanism effectively reduces cellular cAMP levels and leads to a decreased firing frequency of action potentials by nociceptive neurons. This process in turn, is subsequently responsible for reduced pain signaling (240). The existence of at least three different opioid receptor types was suggested, namely MOP receptors, DOP receptors and KOP receptors (231,241,242,243). Organ bioassays demonstrated that morphine-like opioids show a selective high potency to guinea-pig ileum, which is rich in MOP receptors, whereas enkephalins show high potency to the mouse vas deferens, which contains large amounts of DOP receptors (243,244). Recently, a fourth receptor type was identified and was termed as "opioid receptor like – 1" receptor (ORL1; or nociceptin receptor, NOP). The NOP receptor exhibits moderate structural homology with the typical opioid receptors and is currently classified as a member of the opioid receptor family by the International Union of Basic and Clinical Pharmacology (IUPHAR) (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=320&familyId=50&familyType=GPCR). At the cellular level, N/OFQ, the endogenous ligand of the NOP receptor, produces similar actions compared to those of the classical opioids resulting in reduced neuronal excitability (245) and inhibition of transmitter release (246) in animals. Initial studies concentrated on the role N/OFQ and NOP in pain perception. The modulation on pain

by the N/OFQ – NOP system is complex. Under laboratory conditions, N/OFQ was described to induce pro-nociception behaviours and hyperanalgesia when applied supraspinally. In contrast spinally administrated N/OFQ induced analgesic effects (247). Rather than directly acting as a pro-nociceptive agent itself, the possible mechanism of N/OFQ anti-analgesic activity could lie in an interference with the activity of endogenous opioids or that it counteracts stress-induced analgesia during testing procedures in rodents (248). Recently, NOP antagonists have been reported to produce a long lasting analgesia, with similar efficacy compared to morphine (249). Several of those novel NOP antagonisms also demonstrated mood regulation effects as potent antidepressants and/or anxiolytics (250,251), but also prevented the development of morphine-induced tolerance (252) in animal models. In NOP receptor knockout mice, chronic administration of morphine leads to up-regulation of endogenous levels of N/OFQ (253), while selective NOP antagonist also attenuate chronic morphine-induced tolerance in rats (249). Therefore, NOP antagonists may be used as novel analgesics or perhaps be used as adjuvants to reduce the tolerance associated with classical opioids. However, in spite of the structural similarity and similar localization of the NOP receptor with the classical opioid receptors, NOP receptor activity is insensitive to the opioid receptor antagonist naloxone, which is an important discriminatory feature (254). .

Opioid receptors are widely distributed in both the central and peripheral nervous systems, as well as in the digestive tract (255,256). MOP receptors show the highest densities in the forebrain and midbrain of rats, such as the cortex, amygdala, hippocampus, hypothalamic nuclei, raphe medianus, locus coeruleus, sensory trigeminal complex, as well as in the rat spinal cord dorsal horn (257). This distribution suggests their importance in mediating functions such as motor rigidity, autonomic reflexes, endocrine functions, mood and analgesia. At a molecular level, MOP receptors are found in the noradrenergic terminals (258), indicating mood controlling effects of the MOP system via mediating the release of neurotransmitters. For

example, in the rat cerebral cortex, morphine inhibits the release of noradrenaline by directly activating the MOP receptors on noradrenergic nerve terminals (259). MOP receptor agonists also stimulate postsynaptic transmission in the rat hippocampal DG cells by acting on gamma-aminobutyric acid (GABA) interneurons (260). Since dysfunctional GABAergic interneurons and noradrenergic neurons are associated with schizophrenia, bipolar disorder (261) and depression (262), these results highlight the potential of opioids for the treatment of mood disorders such as depression and anxiety (263,264).

In preclinical studies, DOP receptors were mainly found on nerve axons (265). They show high densities in multiple cortex areas, such as the cingulate cortex, frontal parietal cortex, piriform cortex and temporal cortex, whereas in contrast to the MOP receptor, they show much lower densities in the hippocampus, thalamus and raphe nucleus (266).

The KOP receptor is widely distributed in brain regions of pre-clinical animal models such as the amygdala, hypothalamus, thalamus and ventral tegmental area, where they are involved in motivation, mood control, hormone manipulation and stress regulation (267). Unlike the MOP and DOP receptors, KOP receptors show a particularly high expression in the granular layer of the hippocampal DG (249), which supports a potential role in the stress response.

1.2.3.4 Opioids in pain management

Synthesized opioid agonists essentially mimic the activity of endogenous MOP, DOP and KOP receptor ligands (268). Although the binding of opioids to the DOP and KOP receptors involves in the modulation of pain signals, the MOP receptor is the major opioid receptor that responsible for the analgesic effect of opioids (269) (**Table 1**).

Table 1. The distribution of three classic opioid receptors and their endogenous and clinical used exogenous ligands.

MOP: mu-opioid receptor; DOP: delta-opioid receptor; KOP: kappa-opioid receptor; POMC: proopiomelanocortin; PENK: proenkephalin; PDYN: prodynorphin

Opioid receptors	Receptor distribution	Precursors of endogenous ligands	Endogenous ligands	Clinically used ligands		Clinical use
				Agonists	Antagonists	
MOP	cingulate cortex, frontal parietal cortex, piriform cortex, temporal cortex, amygdala, hippocampus, hypothalamic nuclei, raphe medianus, locus coeruleus, sensory trigeminal complex, spinal cord, GI track, respiratory track	POMC	β-endorphin	Morphine, codeine, oxycodone, fentanyl, , tapentadol, pethidine, methadone, dextropropoxyphene, nalbuphine, levorphanol, buprenorphine (partial agonist)	Naloxone, naltrexone (non-selective), butorphanol (partial antagonist), ,	Anesthetics, analgesics for mild to severe pain, cough (codeine), alcohol and opioids dependence (naltrexone), opioids overdose (naloxone), opioid detoxification (methadone), opioids addiction (buprenorphine)
DOP	cingulate cortex, frontal parietal cortex, piriform cortex, temporal cortex, spinal cord	PENK	Enkephalins	None	Naltrexone (non-selective), naltrindole	Opioid overdose
KOP	amygdala, hypothalamus, thalamus and ventral tegmental area, granular layer of hippocampal DG, spinal cord	PDYN	Dynorphins	Bremazocine, nalorphine and pentazocine (weak MOP antagonist/full KOP agonist), pethidine, , nalbuphine	Binaltorphimine	Moderate-to-severe pain, migraine (butorphanol), opioids addiction (buprenorphine)

1.2.3.5 Side effects of opioids

Common side effects associated with opioids include constipation, nausea, itching, drowsiness, loss of appetite, stomach irritation and dizziness. Typically those symptoms already start after 24 – 48 h of opioid use (266). The incidence of postoperative respiratory depression induced by opioids is only found in less than 1 % of patients (270). Even though the risk of developing severe respiratory depression can be reduced in patients by close monitoring, fatal outcomes and severe brain damage still occur even after taking precautions (271). So far, naloxone is reported to be the only treatment for opioid-overdose-related respiratory depression (272). Early animal work suggested that MOP receptors are responsible for the opioid-induced respiratory depression (273), with a differential mechanism compared to the analgesic effects of MOP agonists (274). However, the DOP agonist (+)BW373U86 reversed respiratory depression activity by the MOP agonist alfentanil (275), which suggests that the DOP receptor is able to counteract respiratory depression induced by MOP receptor agonists. Later, the absence of opioid-induced respiratory depression was observed in rats during co-administration of the selective KOP agonist U-50,488H with MOP agonists DAMGO and morphine (276). This illustrates the cross-modulating effects of MOP and KOP receptors in the development of respiratory depression. Apart from the above side effects, long-term administration of clinically used opioids, for example, morphine, also results in behavioral effects such as physical dependence, tolerance and addiction (265,267). It is generally thought that those behavioural side effects of opioids are receptor-type depended (277,278). At present, it is believed that the addictive properties of most clinically used opioids are mainly mediated by the MOP receptor system, because of its motivational and rewarding properties (279,280). Long-term use of MOP agonists causes dependence and tolerance, which leads to decreased therapeutic efficacy in patients (281). Even though DOP agonists have showed some extent antinociceptive and promising antidepressant-like effects in rodents, their pro-seizure and

convulsive properties limit the therapeutic potential (282). Additionally, DOP agonists are showed to reduce gastrointestinal tract motility and cause respiratory depression (283). Activation of the KOP receptor system is able to produce addiction and pro-addictive stress under laboratory conditions (280,281). Mice subjected to stress show dynorphin release and robust KOR activation in both dopaminergic and serotonergic nuclei (284), which suggests a general dysphoric effect of KOP agonists. In addition, the KOP agonist salvinorin apart from its documented antinociceptive effects in preclinical studies, can result in strong hallucination in patients and is therefore also occasionally used as a recreational drug (285). However, this adverse effect seriously prohibits its long-term clinical use as an analgesic (286,287). Along with the development of highly selective DOP receptor opioid ligands, the previously reported roles of DOP receptors have been clarified and novel functions have emerged. Due to the high expression of DOP receptors in the limbic system (288), the DOP receptor system appears to be the most attractive in term of their potential for mood control (289). Furthermore, preclinical data showed that selective DOP antagonists prevented morphine-induced tolerance, suggesting an ability of the DOP receptor to prevent MOP receptor-associated tolerance and dependence (290,291). These novel functions of DOP agonists promise to overcome some of the drawbacks of MOP-specific opioids and open therapeutic indications that have not been associated with the opioid system so far.

1.3 Mitochondria and stress-induced disorders

1.3.1 Mitochondrial dysfunction, cognitive dysfunction and mental disorders

Mitochondria, as the main cellular energy factories, provide ATP for all cellular functions. ATP, the storage molecule of cellular energy, is produced by a series of oxygen-dependent reactions across the mitochondrial inner membrane via the electron transport chain (ETC), which works as sites of oxidative phosphorylation (158). The ETC contains five complexes,

i.e., reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinone-cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V) (138). The major functions of mitochondria are to produce energy, regulate cellular signalling and maintain the synaptic plasticity of neurons (159,160,161). When the respiratory chain is unable to produce adequate amounts of ATP, the neurons of the brain becomes particularly vulnerable due to their high metabolic activity and consequently increased energy demand (162).

On the other hand, mitochondria are also a major source of ROS. ROS are the products of several cellular metabolic pathways and include the highly reactive superoxide anion, H_2O_2 and the hydroxyl radical ($OH^{\cdot}$). These reactive compounds are formed by one-, two- and three-electron reductions of molecular oxygen respectively (292). The majority of ROS are produced at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase) of the ETC (165). Under normal conditions, 1 – 2 % of the oxygen consumed by the cytochrome oxidase is reduced incompletely to form ROS (293). In addition, under physiological conditions, ROS are also generated during normal respiration by the mitochondrial oxidative phosphorylation process (164). Excessive levels of oxygen radicals can damage intracellular proteins, lipids, mtDNA and nuclear DNA (166), which can ultimately exacerbate mitochondrial dysfunction and can lead to neurodegenerative and affective disorders, such as depression and cognitive dysfunction (160,167,168). Therefore, mitochondria represent a valid therapeutic target for pharmacological interventions to treat not only neurodegenerative but also affective disorders. However, at present only very few drugs are in development or are approved that target mitochondrial function specifically.

1.3.2 Oxidative stress, MAO and mitochondrial dysfunction

The mitochondrial enzyme MAO exists as two isoforms (i.e., MAOA and MAOB) with isotype-dependent biological effects (294). Apart from their metabolic function (295), both MAO isoforms are involved in the regulation of behaviour and neuroprotection (296,297). In early animal studies, pretreatment with selective MAOB inhibitors protected the nigrostratal neurons in mice against the damage by neurotoxin and subsequently prevented the development of PD (298). In addition, increased MAOB levels are reported in elderly patients with AD and aged rodents, suggesting a role of MAOB in aging related cognitive decline (299). On the other hand, because MAOA generally metabolizes tyramine, NE, 5-HT and DA (300), MAOA inhibitors are typically used in the treatment of depression (301) and some neurological diseases such as bipolar affective disorder (302) and panic disorder (303). Interestingly, during the metabolic process of monoamines, MAOs also produce ROS in the form of H_2O_2, which is reported to be involved in a range of bioactivities such as cellular apoptosis and DNA repair (304,305). .

Under normal conditions, damage by MAO-generated H_2O_2 is kept in check by endogenous antioxidant systems including H_2O_2 detoxification enzymes catalase and glutathione peroxidase (306). However, under pathological conditions associated with many neurodegenerative diseases (307), MDD (308) and aging (309), the balance between pro-oxidants and antioxidants is altered. Oxidative damage can occur when ROS (e.g., H_2O_2 and $OH^{\cdot}$ radicals) production exceeds cellular antioxidant defenses (310). Likewise, inhibiting MAO activity can reduce the production of H_2O_2 and it was suggested that MAOIs can protect mitochondrial function (311,312). Mitochondria regulate cell survival and cell death through a variety of anti-apoptotic and pro-apoptotic proteins (313). Excess ROS leads to swelling of mitochondria and collapse of mitochondrial membrane potential. Subsequently, mitochondrial cytochrome c is released to activate the caspase cascade, which ultimately triggers cell death

(314). The MAOB inhibitors rasagiline (315) and selegiline (316) have been shown to protect neurons against ROS-induced apoptosis *in vitro* through activating the anti-apoptotic proteins Bcl-2 and Bcl-xL and preventing the release of cytochrome c from the mitochondria. Therefore, it is conceivable that MAO inhibition not only increases monoamine levels to activate membrane receptors but at the same time, decreases the potential of ROS-associated neuronal injury and mitochondrial-dysfunction-mediated cell death. In addition, the novel multifunctional iron chelator and MAO inhibitor M-30 showed inhibitory effects on MAO activity in mouse brains (317), as well neuroprotective effects in neuron cell cultures by inhibiting $OH^{\cdot}$ radical formation (318,319). However, the neuroprotective effect of M-30 was also reported to be independent with its MAO inhibitory activity and could be attributed to its iron-chelating properties instead (320). Interestingly, the activity of MAO is influenced by iron levels in both animals and humans. In rats, iron deficiency is associated with decreased MAO activity (321). In patients with neurodegenerative diseases associated with excess iron accumulation such as Parkinson's disease (PD), Alzheimer's disease (AD) and Friedreich's Ataxia (FA) (322,323,324), increased MAO activity was detected at the sites of neuronal cell death (325). Apart from the MAO-mediated pathway, large amounts of ROS are also produced by several other metabolic mechanisms such as the mitochondrial electron transport chain (293), xanthine oxidase (326), arachidonic acid metabolism (327), and peroxisomal enzymes such as d-amino acid oxidase, fatty acyl CoA oxidase, and urate oxidase (328). Therefore, it cannot be expected that inhibition of MAO activity directly results in lower detectable oxidative stress levels under physiological conditions. However, mitochondrial ROS production via the mitochondrial electron transport chain is directly linked to ROS-induced damage of its components (329) (**Figure 5**). It could therefore be hypothesized that MAOI can protect against localized ROS-damage, which would otherwise lead to excess ROS production

by a dysfunctional mitochondrial electron transport chain, which would be especially prevalent under pathological conditions (**Figure 5**).

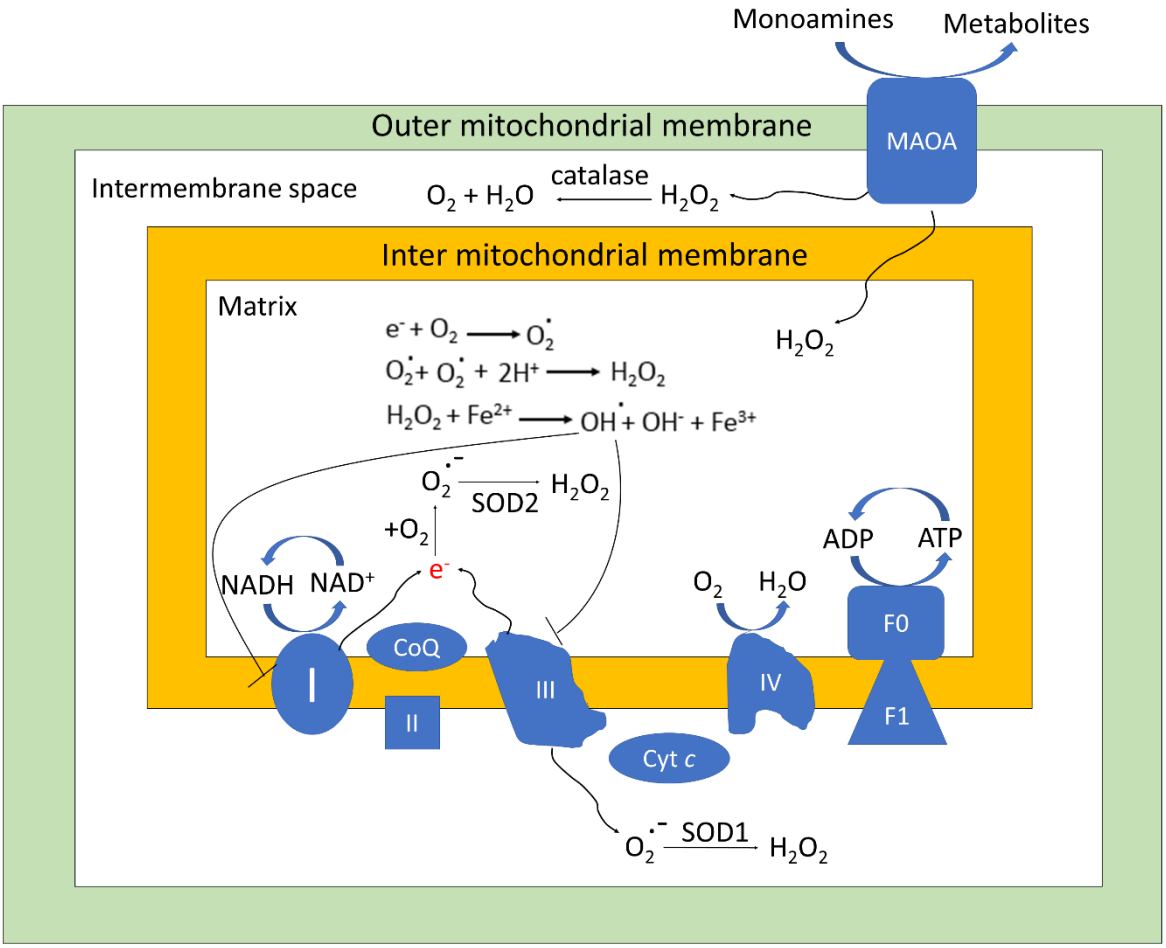

Figure 5. Mitochondrial production of reactive oxygen species.
There are several sources of ROS in mitochondria. The majority of ROS are produced by delocalized electrons at complex I and complex III of the electron transport chain (ETC). The mitochondrial outer membrane protein monoamine oxidase A (MAOA) produces ROS in the form of hydrogen peroxide (H_2O_2) during the metabolism of monoamines. MAOA-produced H_2O_2 is majorly metabolised by catalase in the intermembrane space. A small amount of H_2O_2 penetrates into the mitochondrial matrix where it reacts with ferrous iron. Accumulated ROS cause damage of the ETC and in particular complex I and III, which further increases ROS generation. This leads to lipid peroxidation, mitochondrial dysfunction and apoptosis.

However, given the important role of MAO as one of the main therapeutic targets in depression, a thorough understanding of the relationship between MAO and mitochondrial function promises to unravel the mechanisms underlying neurological disorders that are associated with both systems, but could also lead to the development of improved antidepressants in the future.

At present, this research field has received significant interest and numerous studies are performed to understand the interactions of these two systems in the context of depression (294,330,331). Nevertheless, pharmacological manipulation of MAO and mitochondria and their reciprocal effects still require intensive investigations in the future.

1.4 Novel therapies for mental disorders

1.4.1 Preclinical and clinical antidepressant-like effects of opioids

Although opioids are the first-line therapy for clinical pain management, their antidepressant effects have also been explored over the last few decades (282). However, not much progress has been made towards developing novel opioids as antidepressants or even exploring the clinical use of currently available opioids for the treatment of mood disorders like depression in depth. Despite numerous *in vitro* and *in vivo* studies regarding the effects of opioids on serotonergic and dopaminergic systems, only a few studies have explored the antidepressant potential of opioids in clinical trials (332). Both MOP agonists and KOP antagonists have demonstrated promising antidepressant-like effects in animal models (333,334). Those results prompted the idea to evaluate the antidepressant effect of a combination of a MOP agonist and a KOP antagonist (335), to increase their efficacy. Buprenorphine, as a partial MOP agonist and functional KOP antagonist, draws attention in this context. Interestingly, an early study showed that 0.23 % of postoperative patients that were given buprenorphine experienced euphoria (336), indicating the that the mood-lifting effect of buprenorphine could contribute to the observed analgesic effect in the study. Later, buprenorphine was reported to significantly reduce the severity of depression in elderly patients with treatment-resistant depression within the first 3 weeks of treatment when compared to the placebo group (337). In October 2013, the FDA granted Fast Track status to ALKS 5461, a combination of buprenorphine and samidorphan (a selective MOP antagonist) for the adjunctive treatment of MDD in patients

with an inadequate response to standard antidepressant therapies (338). However, despite of the good safety data of ALKS 5461 (339), there is no reported efficacy data of this combination available so far from phase III clinical trials in patients with MDD (Trial numbers NCT02158533, NCT02085135, NCT02218008 and NCT02158546) by the end of study period (October 2016) which suggests that the trial showed negative results. Given the non-selective nature of buprenorphine, it also remains unclear if a single or a combination of several opioid receptors are involved in mediating the putative antidepressant effects described earlier (336,337). Another example is the atypical opioid tramadol, which is a mixed MOP receptor agonist and a SNRI. Tramadol showed antidepressant effects comparable to venlafaxine, a clinically-used SNRI for the treatment of depression (340). Tramadol was considered the ideal prototype of an opioid-based antidepressant, due to its dual effects as MOP agonist and its presynaptic inhibition of serotonin and noradrenaline uptake (341). However, it was reported that co-administration of tramadol with serotonergic antidepressants could increase the risk of serotonin toxicity which severely limits its clinical use (342).

Although large preclinical studies suggested a strong correlation between the activation on MOP or/and DOP receptors and the increased activity of serotonergic/dopaminergic pathways, the exploration of the antidepressant effects of MOP and DOP receptor agonists in the clinic is still quite slow (343). Until now, only a very limited number of studies have investigated MOP agonists such as oxycodone and oxymorphone in depression (344) (). Similarly, clinical studies on both DOP agonists and KOP antagonists as potential antidepressants are also rare (345).

1.4.2 Importance of developing novel multi-targeting opioids

Chronic use of opioids that mediated by MOP receptor is associated with multiple side effects (346). For example, drugs such as morphine that activate the MOP receptor are mainly used as analgesics in clinic, while their use increases the risk of opioid abuse and dependence (143).

The behavioural effects associated with long-term use of opioids represent great public health concern. Therefore, to achieve optimal analgesic effects while producing minimum side effects, multi-targeting opioids are developed, which act on two or more opioid receptor types at same time. This idea originates from positive treatment outcomes of co-administrating different opioids receptor ligands in both pre-clinical and clinical trials. For example, the MOP agonist morphine combined with the selective DOP antagonist naltrindole produced better analgesic effects and produced less tolerance and dependence compared to morphine alone (144). Similar results were reported for a combination of the KOP agonists U50,488H and the MOP agonist DAMGO in mice (145). Based on these data, the novel bifunctional opioid MDAN-21 with MOP agonism and DOP antagonism effects simultaneously, was reported to be 50-fold more potent than morphine (146). Collectively, these results highlight the possibility of better analgesic outcomes and less adverse effects by multi-targeting opioid ligands.

1.4.3 Bifunctional opioids

Bifunctional opioids, as one typical example of multi-targeting opioids, bind to two different opioid receptors simultaneously (**Figure 6**). This type of ligand can have either the same or the opposing activity towards different receptor types at same time.

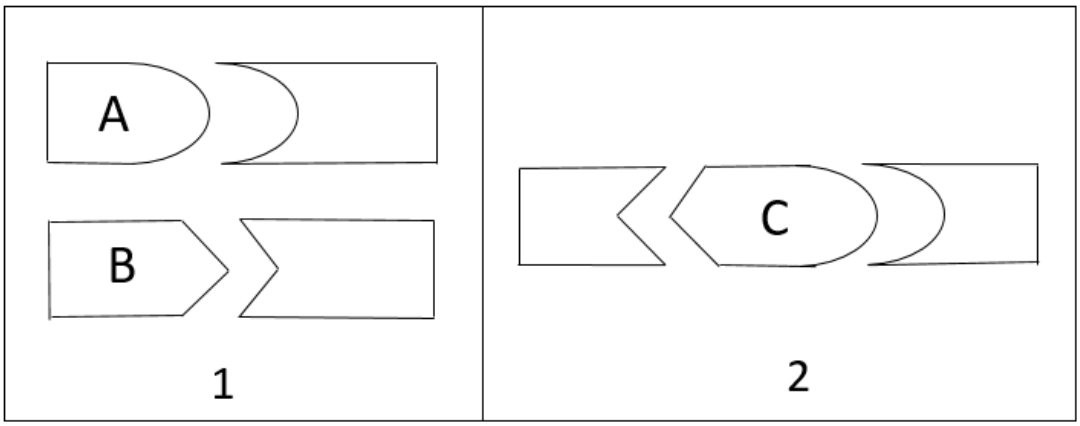

Figure 6. Schematic illustration of conventional opioids and bifunctional opioids.
To achieve the expected therapeutic outcomes, opioids A and B have to be given simultaneously to act on their specific responsive receptors (1). In contrast, the bifunctional opioid C can act on both receptors simultaneously (2).

Opioid agonists with selectivity for any of the three classic opioid receptor types all have limitations because of their side effects and/or weak analgesic activity. For example, morphine is associated with numerous side effects, such as inhibition of gastrointestinal motility, respiratory depression, tolerance and physical dependence apart from the analgesic effect (347), which limits its long-term use for pain management (348,349). The potential clinical uses of DOP and KOP agonists as alternative analgesics are limited by their convulsive and dysphoric effects respectively (350,351,352). So far, there is some evidence that bifunctional opioids with novel biological activities exhibit therapeutic analgesic potential with reduced side effects. This is based on several lines of evidence. One of those is the observation that the selective DOP antagonist naltrindole can reduce the development of morphine-mediated tolerance and dependence in rodents (353). Similarly, blockage of DOP receptor expression can inhibited morphine-induced tolerance and acute dependence in mice (291). On the other hand, chronic administration of morphine upregulates DOP receptor expression in rats (354). Furthermore, chronically administrated morphine was able to retain its full analgesic activity in DOP KO mice without inducing tolerance (355). Taken together, all these observations suggest that a compound that can activate the MOP receptor and block the DOP receptor at the same time could be a promising analgesic with low potential to produce tolerance and physical dependence. In addition, naltrindole has been reported to enhance bowel movement (356) and to reverse respiratory depression induced by the MOP agonist alfentanil (357), which suggests that a bifunctional MOP agonist/ DOP antagonist may also be able to reduce MOP-mediated side effects of slower gastrointestinal motility and respiratory depression. Compounds with this molecular profile (MOP agonist/ DOP antagonist) have been reported. One example is TIPP-

NH_2 (H-Tyr-Tic-Phe-Phe-NH2) (358). However, the development of peptide and non-peptide bifunctional MOP/DOP ligands seems to be difficult due to their poor ability to cross the BBB (359). . In contrast, the bifunctional opioid Dmt^1]DALDA→CH_2CH_2NH←TICP[ψ] that was based on the structure of TIPP-NH_2 , is able to cross the BBB. This molecule showed similar *in vitro* receptor binding characteristics as the parental compound TIPP, and demonstrated similar analgesic effects *in vivo* compared to morphine (360). Non-peptide bifunctional opioids that simultaneously activate the MOP receptor and inhibit the DOP receptor were also investigated. For example, compound SoRI 9409 showed significantly less tolerance development compared to morphine, while its derivative SoRI 20411 demonstrated a 10-fold analgesic potency compared to morphine and less potential to develop tolerance (361,362,363). Interestingly, bifunctional opioids that block the MOP receptor while activating the KOP receptor may have the therapeutically potential to treat cocaine abuse. Cocaine-associated abuse is partially mediated by increased extracellular dopamine levels in the mesolimbic dopamine system (364,365). Both MOP antagonists and KOP agonists inhibit mesolimbic dopamine release in animals (366,367), suggesting a positive effect of bifunctional MOP/KOP ligands in cocaine abuse. However, no bifunctional molecules that target the MOP/KOP recptors have been synthesized so far to test this hypothesis.

Overall, only a few bifunctional ligands have been investigated with limited success. This is mainly attributed to their poor bioavailability, polarity and/or poor stability (368). However, with an increased understanding of the opioidergic systems and the promising therapeutic and adverse effects profiles of bifunctional opioids, it can be anticipated that the development of novel opioids for both pain management and mood control will continue to be explored.

1.4.4 Mitoprotective therapies

Mental disorders and cognitive dysfunction are associated with mitochondrial dysfunction (369,370). Therefore, protecting mitochondria from oxidative damage and restoring mitochondrial function can also be envisaged as an effective therapeutic strategy for the prevention of mental disorders and cognitive deficits. This field is still in its infancy and drugs that are specifically designed to restore and protect mitochondrial function have just entered the market. The first drug that was recently approved in 2015 by the European Medicines Agency for the treatment of a mitochondrial disorder is the short-chain quinone idebenone and several similar drug candidates are in phase II and III of clinical development. Theoretically, drugs such as idebenone that have potent antioxidant and mitoprotective effects could be repurposed for the treatment of depression and cognitive impairment.

1.4.4.1 Idebenone: overview

Idebenone (2-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-cyclohexa-2,5-diene-1,4-dione) is structurally similar to Coenzyme Q10 (CoQ10), but displays very different pharmacological characteristics (371,372). Idebenone was first synthesized by Takeda Pharmaceuticals in the 80's and successfully developed for the treatment of Alzheimer's disease (AD) as well as learning and memory deficits, although its specific molecular activity at this point was largely unknown (373). Idebenone can cross the BBB, is well tolerated in patients with AD (374) and has a favorable safety profile (375,376). Idebenone has multiple molecular activities that include a modulation of mitochondrial respiration, potent antioxidant function and cytoplasmic-mitochondrial electron carrier function (372,377,378,379). Moreover, idebenone showed neuro-protective properties by maintaining mitochondrial membrane potential, improving the activity of the respiratory complexes and cellular energetic levels and inhibiting lipid peroxidation (375,380,381). However, in human subjects, idebenone is absorbed rapidly

and reaches peak circulating concentrations in 15 minutes in a dose-dependent manner, but it is also rapidly excreted. In rats, the plasma half-life of idebenone was reported to be only 30 minutes, which is believed to be the result of a high first pass effect (382). Despite these unfavorable pharmacokinetics, which complicate a rational understanding of its molecular mode of action, protection of neurological function by idebenone has been reported in many pre-clinical models and clinical trials (383,384). Parallel to the protective effect of idebenone against neurotoxin amyloid β-peptide (Aβ) in rat hippocampal neuronal cells (385), 20 mg/kg idebenone ameliorated Aβ-induced working spatial memory impairment in rat models (386). In addition, degeneration of cholinergic neurons is reported to be associated with impaired cognitive function in both aged animals and humans (387,388) and nerve growth factor (NGF) seems to play a central role in the survival of cholinergic neurons (389). Twenty mg/kg oral administrated idebenone increased NGF content in the frontal and parietal cortex of aged rats, and boosted the activity of the cholinergic marker enzyme choline acetyltransferase in striatum to the level of young rats (390). In addition, protection of cholinergic neurons and restoration of cognitive function by systemic treatment with 10 – 30 mg/kg idebenone were reported in other studies (391,392,393). In5 out of 6 large randomized placebo-controlled clinical trials, idebenone was able to improve symptoms of neurodegenerative diseases (e.g., AD) at rat equivalence doses. For example, a single dose of 120 mg idebenone per day (approximately 1.71 mg/kg based on a 70 kg person and equivalent to 10.63 mg/kg in a rat of 300 g, based on FDA-suggested animal-human equivalence dosing) for 6 months in AD patients showed positive treatment effects in improving the cognitive dysfunction (394). In another study, 120 mg idebenone (tid) (approximately 5.14 mg/kg/day based on a 70 kg person and equivalent to 31.89 mg/kg/day in a rat of 300 g) provided symptomatic improvements in patients with AD, showing better memory and more stable mood state (395). At a lower dose, 45 mg idebenone (tid) (approximately 1.93 mg/kg/day based on a 70 kg person and equivalent to 11.96

mg/kg/day in a rat of 300 g) also reversed attention and memory deficits in patients with Alzheimer's – type dementia in only 4-month treatment (374). However, one study reported that idebenone was ineffective in slowing the decline of cognitive function in patients with AD, after a 12-month treatment at doses up to 360 mg per day (approximately 5.14 mg/kg based on a 70 kg person and equivalent to 31.89 mg/kg in a rat of 300 g), in spite of the stated good safety profile in the study (396). Unfortunately, this was the study sponsored by Takeda Pharmaceuticals that was intended to be used for acquiring market authorization. At present is is still unclear why this single trail failed to show a protective effect. Nevertheless, the majority of pre-clinical and clinical trials suggest that idebenone has the potential to improve cognitive function and mood state. Even though it is technically difficult to measure neuronal survival in humans directly, the demonstrated positive effects of idebenone in other neurodegenerative diseases such as Leber's Hereditary Optic neuropathy (LHON) (397,398), indicate a potential protective role in preventing neuronal death in patients, which can likely contribute to the observed symptomatic improvements.

1.4.4.2 Idebenone: potential in mood control

The antioxidative effects of idebenone have been widely described. Idebenone decreased non-respiratory oxygen consumption via inhibiting lipid peroxidation, membrane lysis and mitochondrial swelling in rat mitochondria (399). This drug exhibited nearly 7 times higher antioxidant capacity than vitamin E in an *in vitro* assay (400). In glutathione (GSH)-depleted neuronal cells, 1 μM idebenone significantly improved cell viability, demonstrating neuro-protective effects (401). *In vivo*, idebenone decreased lipid peroxidation and increased catalase levels as well as several endogenous antioxidants (402,403). In rat brain synaptosomes, idebenone showed similar effects as vitamin E to prevent oxidative stress and mitochondrial swelling induced by a ROS-inducing compound (404). Importantly, in the context of

depression and cognitive dysfunction, idebenone was also reported to increase 5-HT turnover and concentrations of its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in brain regions such as the HPC, hypothalamus and cerebral cortex that are important for mood control (405). There is some evidence to suggest that depression could be related to increased oxidative stress (406). The resulting elevated levels of ROS are likely to damage lipids, proteins and DNA, which ultimately leads to impaired mitochondrial function (407). In addition, lower plasma levels of the 5-HT precursor tryptophan and abnormalities in 5-HT function are reported in patients with depression (408,409). Since idebenone has shown promising antioxidant activity and abilities to correct 5-HT abnormalities, idebenone might be tested as potential antidepressant in future. In a clinical trial, idebenone improved psychiatric symptoms through increased 5-HT turnover in dementia patients (410). In addition, idebenone treatment improved psycho-physical wellbeing in patients with chronic cerebrovascular disorders without any severe adverse effects (376). Parallel to the clinical findings, idebenone exhibited antidepressant-like effects similar to the clinically used tricyclic antidepressant imipramine in a rat model of depression (411).

1.4.4.3 Idebenone: learning and memory deficits

Recent evidence suggests that mutations of mtDNA are associated with neurodegenerative syndromes and stroke, indicating a crucial role of mitochondria in age-dependent cognitive dysfunction (412,413). Accumulated oxidative stress and altered mitochondrial ETC function has also been reported in patients with AD (414,415). What's more, idebenone reportedly improved cerebral energy metabolism and increased the levels of nerve growth factor, which are two important factors related to neuronal cell death and memory loss seen in AD patients (391,416).

Idebenone significantly improved cognitive performance test in patients with Alzheimer type dementia in multiple studies (374,395,417,418,419). In pre-clinical rat studies, idebenone improved short-memory that was impaired by 5-HT deficiency (393). Similarly, in mice with impaired memory due to amyloid β-peptide induced neurotoxicity, idebenone prevented learning and memory deficits (420). Collectively, idebenone appears to improve age-dependent cognitive function by normalizing the neurotransmitters system and ameliorating energy failure due to of oxidative damage.

1.5 Animal models of mental diseases

Animal models have been utilized to investigate human diseases for many years (421). Even though the validity of preclinical models for the study of mental disorders are still questioned by different research groups, their use has significantly increased our understanding of specific molecular pathologies and also led to the development of novel treatment options for many chronic diseases such as asthma, tuberculosis, meningitis and breast cancer. In addition, animal models also increased our ability to successfully model complex diseases in different animal systems (421,422). To assess the effectiveness and efficiency of an animal model of a human disease and to use this model to evaluate drug effects across laboratories, three validity criteria were initially introduced by McKinney and Bunney in 1969 (423).

1.5.1 Validities of animal models

Validity in its definition is a criterion that describes the reliability of a pre-clinical model in terms of representing mechanisms of diseases, mimicking drug treatment and its ability to predict therapeutically outcomes in the clinic. The validity criteria for depression models has been widely discussed and altered by many researchers in the last 30 years (184). At present, the validities introduced by Wilner et al in 1984 are widely accepted and include face validity,

construct validity and predictive validity (185). The presence of these criteria in an animal model provides confidence in the model to accurately reflect the human disease state and is essential for comparison across studies from different laboratories.

Face validity

Face validity requires that the symptoms observed in an animal model replicate clinical features of the disease (186). In relation to an animal model of depression, the model should be able to include one or several (ideally all) key clinical symptoms. These include failing to escape adverse stimuli (learned helplessness), reduced interest towards reward (anhedonia), sleep disturbances, decreased libido etc. However, the efficiency of some models, which only requires one or two days to produce these symptoms, are questionable when the slow time course of developing depressive symptoms in patients is taken into account (187).

Construct validity

Construct validity refers to the observation that the experimental conditions of the animal model can replicate the cause of disease in patients (186). To achieve this validity, researchers can re-construct the etiological processes that causes a disease in human patients. Since exposure to stress is one of the major causes of depression (29,187,188,189), exposing the testing animals to stressful stimuli such as shocks and forced cold-water swimming is expected to generate depressive-like symptoms in rodents. In addition, another direct method to achieve construct validity is to knock-out known disease-causing genes, as long as the resulting animal phenotype accurately reflects the human disease state (190).

Predictive validity

The final criterion centers on the assumption that the animal response to a drugs can predict the therapeutic efficacy of a potential drug in patients (186). Predictive validity examines the use of therapeutic options of a pharmacological nature and their effect on the symptoms in the model (184). An accurate model should be able to demonstrate comparable efficacy of a drug in the model compared to a clinical trial (191).

1.5.2 Animal models of depression

It is widely known that chronic stress is associated with the onset of depression (26,424,425). There is significant evidence that most episodes of depression are likely the consequence of prolonged exposure to a stressful environment (13,14,15,16). Chronic or lifetime stress is a strong predictor for the development of depressive symptoms (17), and is associated with pathophysiological changes in brain function and structure. Stressful situations over extended periods of time can lead to reduced hippocampal size, a brain area that is specifically involved in the regulation of mood in both animals and humans (426). This strong link between stress and depressive symptoms has been used as the cornerstone of creating animal models of depression, which are vital for the study of this disorder as well as for research into novel antidepressant treatment options.

1.5.2.1 Classification

A number of pre-clinical models are currently used to evaluate the pharmacological effects of potential antidepressants (427). These models have been evaluated on the basis of above three major criteria, namely construct validity, face validity and predictive validity (428). The more valid a particular animal model is, the more accurate and reliable is the data it produces. Current

animal models of depression can be generally classified into four main classes based on the nature of their induction phase, as shown in **Table 2**.

The first class of animal models of depression is based on the application of acute or sub-chronic stressors for the induction of depressive-like symptoms. It includes behavioral models such as the FST and the tail suspension test (TST), which are also called despair-based models. They are frequently used in fast drug-screening studies of novel antidepressants, because of their easy and economical nature, as well as their acceptable face and predictive validities (13,429,430). However, these models lack the essential construct validity and only produce limited short-lasting depressive-like symptoms (430). The LH model of this class is probably the only one that stands out, since it offers stronger validation and medium-term lasting behavioral and related cognitive symptoms. Nevertheless, the electrical shocks that are used in this model and the comprehensive equipment that is required to establish this model limit the use of this model.

The second class of animal models of depression is based on long-term exposure to various stressors to trigger the manifestation of depressive-like symptoms such as anhedonic-like behavior, aversion towards activity and changes in appetite etc. It includes three main animal models of depression: the chronic mild stress (CMS), the chronic social defeat (CSD) and the chronic social isolation (CSI) models. The CMS model demonstrates particularly strong validities, in term of generating anhedonic-like symptoms in stressed animals (431). However, anhedonic-like behavior, a crucial index of depressive mood in this type of models, is not only specific to depression but also seen in schizophrenia and substance withdrawal (432,433,434).

The third class of pre-clinical depression models applies various biochemical and pharmacological concepts to mimic clinical observations. The models in this class are named according to the pathophysiological and molecular pathways of depression that they manipulate. They manifest either potential molecular sources of the depressive symptoms or

adaptive/responsive mechanisms of depression, such as the function of the HPA axis (435,436,437). In rodents, retinoid receptors are concentrated in limbic areas that have been associated with depression, including the amygdala, the prefrontal cortex, and the hippocampus (438). Retinoids are known to influence neurotransmitter systems that have been associated with depression, in particular dopamine but to some extent also serotonin and norepinephrine (439). This connection is supported by clinical observations that acute depressive symptoms have been associated with retinoic acid therapies (440). This provides a causal association between increased level of retinoids and the development of MDD. In addition, impaired immune function and hypersecretion of cytokines such as IL-1β, IL-6 and γ-IFN have been observed in patients with MDD (441,442). In a pre-clinical model, IL-1 injection induced chronic immobilization under mild stress condition, which is indicative of a worsening of depressive symptoms (443). Therefore, this third class is not only designed to generate depressive symptoms, but also serves as a tool to study the pathophysiology of depression and the involvement of particular molecular pathways. However, the major limitation of these models is that they are associated with a wide range of behavioral abnormalities, which may be not be specific to depression (444,445).

The fourth class of animal models of depression involves the application of genetic and surgical techniques, which can permanently change animal phenotypes and behavior. Those models include olfactory bulbectomized rodents (446,447), genetically modified strains such as the stress-sensitive Flinders rat (448) and specific receptor-knockout mice that show depressive-like symptoms (for a thorough review on genetically modified mice strains used as animal models of depression see Cryan and Mombereau's study (449)). Although these animal models are particularly useful when studying specific aspects of the pathophysiology and pharmacology, they offer very poor construct validity compared to other classes. Generally, models of secondary depression and the immutable models of depression are not only able to

produce various stress-induced symptoms, but also can be used to study depression-induced cognitive changes by combining them with models of learning and memory (450). In addition, these pre-clinical models can be used in combination with other rodent models of depression, such as the FST (451). These combinations can help to investigate the onset mechanisms of depression without inducing additional physical stimuli.

Table 2. Classification of current animal models of depression and description of their main pros and cons as described in the literature.

FST: forced swimming test, TST: tail-suspension test, LH: learned helplessness, CSI: chronic social isolation, CSD: chronic social defeat, CMS: (unpredicted) chronic mild stress

Main Classes	Models	Stressor	Main Advantages	Main Disadvantages	Example references
Acute and sub-chronic stress-induced	FST	Inescapable forced swimming	Fast induction and drug-screening, cheap & easy setting	Unspecific response to non-antidepressants, weak validities, present single symptom	(429,452,453)
	TST	Tail suspension			
	LH	Inescapable electric shocks	Strong validation, variety of behavior & symptoms	Comprehensive protocol & equipment, strong stressors	(429)
Chronic stress-induced	CSI	Prolonged-chronic isolation	Strong validity, variety of depressive symptoms	Long experimental duration, complex setting, anxiety symptoms	(454)
	CSD	Repeated bouts of social subordination			
	CMS	Chronic expose to alternate and variable stressors	Strong validity, long lasting symptoms	Long experimental duration, anxiety symptoms	
Models of secondary depression	HPA axis dysregulation	Administration of corticosterone	Correlation with pathophysiological and molecular mechanisms of depression, present various depressive symptoms	Questionable correlation with depression	(435,455,456,457)
	Retinoic acid model	Prolong use of retinoic acid			
	Immune system dysregulation	Administration of pro-inflammatory cytokines			
Immutable models	Olfactory bulbectomy	Surgical removal of olfactory bulb	Variety of symptoms, specificity in studies of particular pathways	Indistinguishable adaptation mechanisms	(446,458)
	Genetically modified models	Genetically selected for hypersensitivity to drugs, receptor knockouts etc.			

1.5.2.2 The most frequently used animal models of depression

The three most popular and widely used pre-clinical models of depression are the FST, the LH and the CMS model. Bibliometric data produced from analytical searches using the PubMed database between 1980 and 2016 reveal increasing numbers of publications using these models, to varying degrees (**Figure 7**).

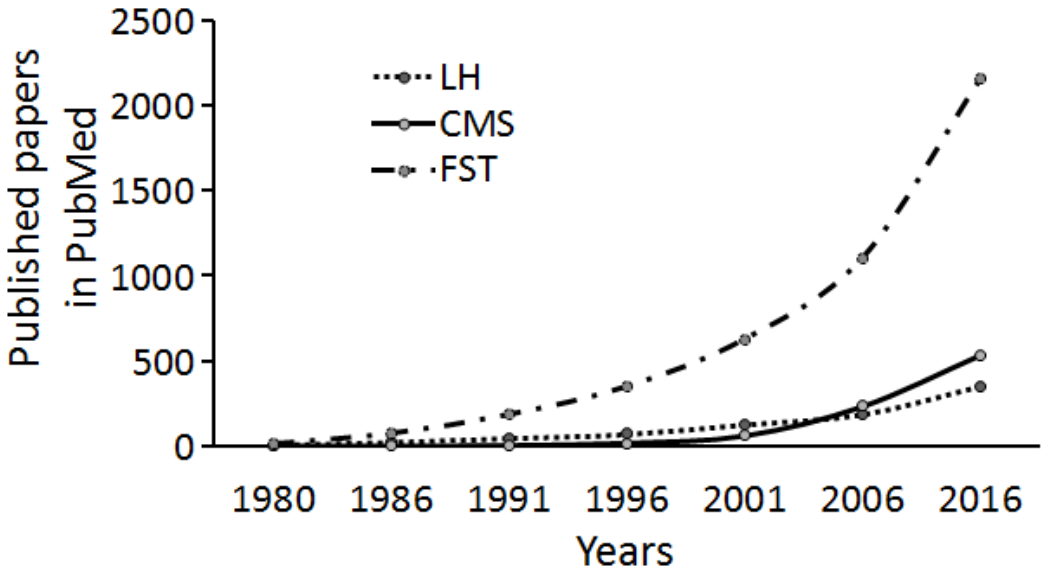

Figure 7. Cumulative number of original research articles published from 1/1/1980 to 31/12/2016 listed in PubMed that used the forced swimming test (FST), the learned helplessness (LH) or the chronic mild stress (CMS) as primary models of depression.

The FST model appears to be the most 'popular' model in pre-clinical depression research (70.9 % of the total published papers using this animal model of depression) and shows the steepest increase in published articles among the three models, probably due to its low set-up cost, simplicity and short experimental duration. On the other hand, the powerful validity and the manifestation of long-lasting symptomatology are probably the strong points of the CMS model that make it the second most commonly used model. Finally, the number of published articles using the LH model has shown an approximate 5-fold increase compared to the published papers in 1996. Clear advantages and disadvantages are inherent to all three models, which will be discussed below along with the technical variabilities of these models.

Although it generally accepted that the documented variation of face validity in these models could be explained by their conceptual and methodological differences, it appears that substantial differences in behavioral responses are also observed between studies that use the same animal model, as shown in **Table 3**. For example, the use of specific strains, such as the stress-sensitive Flinders rats, can be beneficial to obtain better readouts and reduce variance for selected behavioral paradigms. Nevertheless, differing results are also reported for studies using the same animal strain. In addition, small alterations of methodological protocols contribute to the divergent observed animal responses in different studies. Due to the highly technical nature of these behavioral models, research groups tend to base the technical parameters of their experiments (such as time length of induction, intensity and duration of stressors, types of observational arenas, modes of measurement etc.), on their research needs and experimental observations. Thus, there is a huge number of protocols in use, which amplifies variations in animal responses.

Table 3: Examples of antidepressant effects in three stress-based models of depression using different animal species and strains.
The reduction of depressive-like symptoms in experiment groups are presented as percentages of the stressed-saline group.

Model	Recorded behaviours	Animal species & strains	Tested antidepressants	Dose (mg/kg/day)	% reduction of depressive-like symptoms in experiment group	Reference
FST	Immobilization during monitoring phase	Wistar rat	imipramine	15, i.p., 22 days	-82.4%	(459)
		Wistar rat	imipramine	15, i.p.	-25.0%	(460)
		Wistar rat	imipramine	15, i.p.	n/a	(61)
			phenelzine	10, i.p.	-62.5%	
		NIH Swiss mouse	imipramine	10, i.p.	-43.3%	(461)
		NIH Swiss mouse	desipramine	20, i.p.	-60.0%	(462)
		C57/BL6 mouse	desipramine	20, i.p.	-15.4%	
		Swiss mouse	tranylcypromine	4, i.p.	-13.0%	(463)
			phenelzine	4, i.p.	+4.0%	
	Immobilization counts	Sprague Dawley rat	fluoxetine	10, i.p.	-31.3%	(464)
		Sprague Dawley rat	desipramine	20, i.p.	-45.5%	(465)
		Wistar-Kyoto rat	desipramine	20, i.p.	-34.9%	
LH	Escape failures	Wistar rat	imipramine	15, i.p., 22 days	-45.5%	(466)
		Wistar rat	imipramine	32, i.p.	n/a	(467)
		ICR mouse	desipramine	10, p.o.	-52.9%	(468)
		CD rat	fluoxetine	50, p.o.	n/a	(469,470)
			imipramine	50, p.o.	-68.2%	
	Escape latency	Sprague Dawley rat	escitalopram	10, i.p.	-40.9%	(54)
			desipramine	10, i.p.	-45.5%	
		Swiss mouse	fluoxetine	30, i.p.	-43.5%	(471)
CMS	Amount of sucrose consumption	Wistar rat	imipramine	10, i.p.	-46.2%	(472)
		Wistar rat	imipramine	10, p.o.	-54.5%	(473)
		SD rat	fluoxetine	10, i.p.	-60.0%	(474)
		Wistar rat	imipramine	10, i.p.	-57.8%	(475)
	Preference of sucrose solution to plain water	Wistar rat	imipramine	10, i.p.	-24.7%	(476)
			fluoxetine	10, i.p	-21.9%	
		Wistar rat	fluoxetine	10, i.p.	-10.8 %	(477)
		C57BL/6 mouse	imipramine	20, i.p.	-12.4%	(478)
		C57BL/6 mouse	imipramine	30, p.o.	-30.0 %	(479)

1.5.2.2.1 The forced swimming test

The FST was originally described as “a new method for inducing a behavioral state in rats that resembles depression” (480). This model is described to induce a low-mood state in rodents, so-called “behavior despair”, in a fairly short period of time. Because only limited time is needed to induce depressive-like symptoms in this model, contrary to the development time of clinical depression in patients, the FST is now only seen as a quick tool to screen for potential antidepressants (481,482). Nevertheless, at present, it has become the most widely used pre-clinical model to assess antidepressant activity, due to its ease of use and its ability to predict a broad range of antidepressant activities (483). In this model, the rodents are placed into an inescapable cylinder with cold water and are forced to swim. The time of immobility is used to define the floating of the animal when in the water, without active swimming but only necessary minor movements required to keep the head above the water. Immobility is used as the predominant index of the level of behavioral despair and, therefore, increased duration of immobility are characteristics of depressive-like behavior in this model.

The original description of the rat FST procedure contained two phases (480). Initially, rats received a 15 min forced swimming training, followed by a 6 min testing session 24 h after the training (**Figure 8**). A mouse FST model was modified by using only a single 15 min session for testing the efficacy of potential antidepressant drugs to reduce immobility levels (484).

Figure 8. General protocol of the forced swimming test (FST).
Animal training (conditioning to the circumstances) is followed by a single day interval before re-exposure to the experimental setting, where after habituation behaviour is recorded. Main behavioural measurements include immobility times and counts of specific time-blocks of continuous activity (i.e. climbing, swimming)

Because of its simplicity, this paradigm is considered the most suitable for high-throughput screening of antidepressant compounds in rodents. On the other hand, the FST can also be used after chronic stress exposure (485,486), as a follow-up method to measure the development of depressive-like symptoms in a chronic-stress-based model and to quickly evaluate drug activity (487). This feature promotes the use of the FST not only as a model *per se* to predict antidepressants activity, but also as an assessment of depressive-like symptoms that have been induced by other depression paradigms. Recent modifications were introduced based on different observed activities, as well as differences in the behavioral pharmacology among drugs. Active swimming, such as diving, climbing and swimming are now also routinely measured individually (488). In addition, serotonergic and noradrenergic antidepressants differentially influence the swimming and climbing behavior in this model. The serotonergic system mediates the swimming motion, whereas noradrenergic antidepressants enhance the climbing behavior (active movements with forepaws in and out of water, usually against the cylinder wall) (488), which suggests that it might be important to distinguish these behaviors when using this model. Both TCAs (489) and SNRIs (490,491) increase climbing and swimming activities. Because increased locomotion can be confused with decreased immobility as an index of behavioral despair, most FST studies are combined with the open field arena (e.g. open space that records movement) to assess potential hyperactivity. All clinically used antidepressants show efficacy in the FST without affecting locomotion in the OF test, compared to the immobility scores and locomotion of non-treated controls (492), which indicates that the swimming, climbing and diving behaviors in the FST can be increased without inducing hyperactivity. Although the FST is used for nearly three decades, its strength of validity is still disputed (493). Recent modifications to this model are implemented either to increase sensitivity and specificity to the treatment, or to improve the consistency of results between studies or research groups. **Table 4** summarizes the main differentiating parameters

among studies using the FST as a model of depression. Even simple model parameters such as the cylinder diameter, the depth of water and the water temperature are sensitive enough to lead to variations in measured responses (494). For example, a smaller cylinder diameter has been accused of generating more false positive responses due to the animals' rotatory locomotor activity (495), which is another argument for the inter-validation of the FST measurements with a locomotion test like the open field. On the other hand, water depth is one of the predominant differences among studies. In the rat model of the FST, a water depth of 30 cm was reported to produce more "behavioral despair" (496), compared to a depth of 15 cm that was used in the original protocol (480). The depth of the water should be sufficient to lead to non-supporting swimming in relation to the rats' size and full leg extension. Finally, both high and low water temperatures can result in short-lasting immobility and false-positive results, suggesting that water temperature should be ambient for optimal results (497,498).

Table 4: Examples of variability in major methodological parameters in the forced swimming test protocol.
Studies in mice show a higher degree of variability in the protocol parameters than rat studies. Training session duration and testing duration show the largest variability. Up to date, there are no analysis that document an advantage of a particular parameter value over another in terms of model validation. Nd: not described in the study

Training duration (min)	Animal Species	Cylinder size		Water level (cm)	Water temp. (˚C)	Testing duration (min)	Main measurements of recorded behavior	Example references
		Height (cm)	*Diameter (cm)*					
0	CD mouse	16 - 25	10	6 - 15	21 - 23	6	Total duration of immobility during the last 4 mins	(484) (499) (500)
	ddY mouse							(501)
	C57/BL6 mouse							(502)
15	C57BI/6J mouse	*Nd*	*Nd*	*Nd*	~ 30	4 trials x6 mins + 7min inter-trial interval		(503)
2 trials x10min + 24h inter-trial interval	ICR mouse	*Nd*	*Nd*	15	~ 23	6		(504)
15	Swiss mouse	18.5	12.5	13.5	25 ±1	5	Total duration of immobility	(505) (506)
0	SD rat	46	20	30	25 ±1	15	Counts of immobility, swimming and climbing	(507,508,509,510,511)
15	SD rat	40 - 46	18 - 20	30	25 ±1	5	Total duration of immobility	(480) (512)
15	Wistar Kyoto rat	46	20	30	24 - 26	5	Total duration of immobility, swimming and climbing	(513)
	Wistar rat							(514)

1.5.2.2.2 The learned helplessness model

One of the core symptoms of clinical depression is the feeling of helplessness, which manifests in the form of losing any meaning in life and giving up trying to escape from a stressful situation, as a result of exposure to uncontrollable events (515). The features of learned helplessness are highly translational from mammals and even non-mammal species to humans (516). The first LH paradigm was described in dogs (517), where a series of unconditioned stimuli, namely mild electric shocks, were used to induce depressive-like symptoms. Clinically, learned helplessness refers to a mental state in patients that 1) fail to control unpleasant stimuli, and 2) lose the willingness or ability to avoid future stressful events (518). Problem solving is a typical example to explain this behavior. When people fail to fulfill specific tasks, they believe that they are not capable to solve similar tasks and consequently they generate negative expectations regarding any future attempts in a similar task. This passive state of mind contributes to poor performance, and leads to the manifestation of learned helplessness (515,519).

Similarly, rodents exhibit changes in their emotional and cognitive status, as well as significant performance deficits in behavioral tests (520). Taking advantage of rodents' adaptive ability to avoid stressors and danger, inescapable shocks (IS) are used in this model to produce the "helplessness" symptoms. After learning the uselessness of their positive avoidance response to the stimulus, a negative coping strategy, called escape failures, is generated in the following test phases (521). The LH protocol as a depression model includes three phases, the induction of depressive-like symptoms, the recovery and the test phase (**Figure 9**). In the induction phase, LH symptoms are induced by delivering inescapable electric foot-shocks (429,522) or tail-shocks (523,524). The induction is typically composed of 60 trials that each includes a stressor-delivery period and an interval period. After a recovery period of at least 24 hours (to allow memory consolidation), the animal enters the final phase (testing) where they are presented

with escapable electric shocks. In this scenario, the animals are allowed to exit through an opening to a neighbouring safe chamber. The shocks are delivered acutely (3 s) followed by a half-minute interval, with this trial cycle repeated 30 times per day, for 3 consecutive days. Non-induced animals will immediately seek the available exit when presented with mild electric shocks, in an effort to avert the stressor (active avoidance). Learned helplessness-induced animals though will not seek to exit but rather accept the "inevitable" stressor (a behaviour called "escape failure"). The measurable response in the LH model is the number of escape failures during the test phase, in each of the 3 days. In order to differentiate the active avoidance responses of the animal (exit during shocks) with passive avoidance responses that account for anticipation, the model includes the use of a short unconditioned stimulus immediately prior to the shock delivery during the test trials (usually a tone or light). Measurement of the number of escape failures, passive avoidance responses and number of escapes during the inter-trial interval time, are used by the model as surrogate markers of depression, instrumental learning and locomotor activity respectively (525,526).

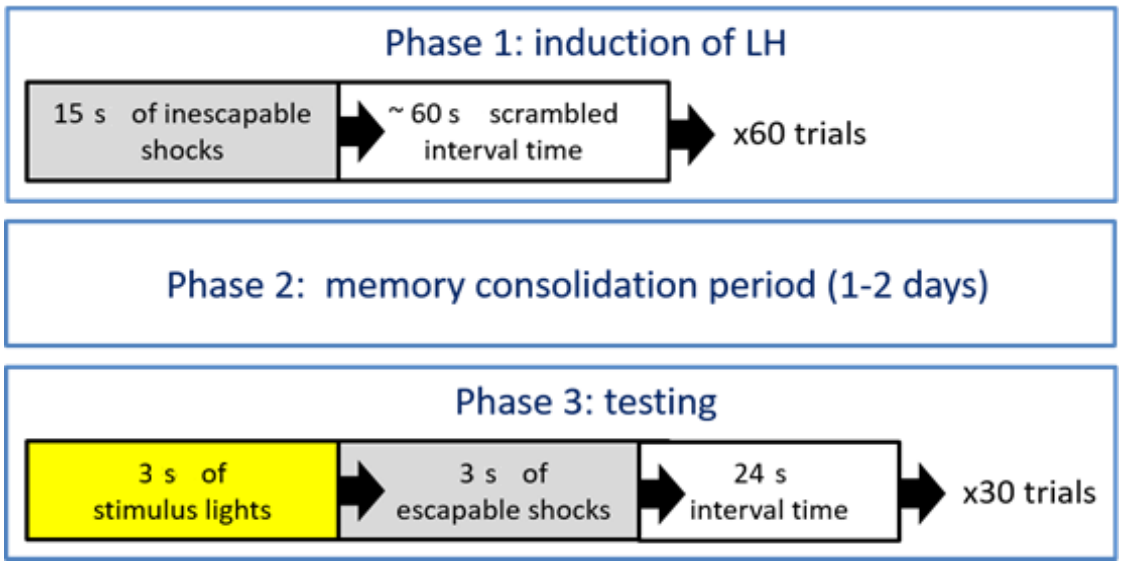

Figure 9. Structure of the most frequently used protocol for the learned helplessness model, which is divided in three phases.
Phase 1 (induction) provides repetitive trials of inescapable shock periods intervened by a short recovery period. Phase 2 offers a long rest period for memory consolidation, whereas

Phase 3 is the testing phase which involves repetitive ES trials intervened by short recovery periods. The escape failures of the animals are recorded as surrogate marker of depressive like symptoms.

Unlike the FST and the CMS models, only a limited number of studies have looked at the impact of protocol variability on the efficiency of the LH model. It was suggested, that the parameters that define the electric foot-shocks (i.e. intensity, duration, delivery pattern etc.), are most vital for a successful induction phase (527,528). Apart from the definition of the electric shocks (intensity and duration), there are a number of numerical parameters that describe the number of sessions, the number of trials, the length of intervals, as well as the use of conditioned and unconditioned stimuli during the test phase (**Table 5**). Although there are not many studies that focused on the effect of these parameters for the efficiency and validity of this model, it is important to note that the nature of the LH model provides the ability to use specific internal controls to demonstrate the effect of some parameters in the measurable response. For example, an animal group that receives ES during the induction phase, instead of IS provides the internal benchmarking for determining the effect of IS on the model (529,530). Nevertheless, new studies that can provide more detailed studies on the connection between the LH parameters and the manifestation of depressive-like symptoms are urgently needed, in order to assess the LH model and improve it further.

Table 5. Different protocols used in the induction phase and testing phase of learned helplessness model.
Key influence factors, namely number of sessions of each phase, number of each session, intensity and duration of shocks and the duration of intertribal interval are listed as index of per protocol

	Training sessions	Trials per session	Shock (mA)	Shock duration (s)	Intertrial interval (s)	Interphase interval	Testing sessions	Trials per session	Trial type	Trial duration	Shock (mA)	Intertrial interval (s)	Reference
Wistar rat	1	60	0.8	15	60	2	3	30	CS/US	3s light + 3s shock	0.8	24	(525,531,532)
Wistar rat	1	60	1.0	2	30	1	1	40	US only	Maximum 30s shock	1.0	Average 60	(533)
SD rat	2	90	2.0	9.9	2, 5 or 10	2	2	50	CS/US	5s light & tone + 5s shock	2.0	30	(534)
SD rat	2	60	0.65	30	20 - 40	1	2	30	CS/US	3s tone + 6s shock	0.65	Average 30	(535)
Swiss-Webster mouse	1	14	0.28	10s light + 20s shock	20	1	1	10	US only	2s shock	0.28	60	(536)
Balb/c mouse	2	30	0.3	10s light + 20s shock	25 - 35	1	3	30	CS/US	10s light + 20s shock	0.3	Average 30	(537)
FVB/N mouse	2	180	0.15	1 – 3	1 - 15	1	1	30	CS/US	5s light + max 10s shocks	0.15	30	(538)

1.5.5.2.3 The chronic stress model

The CMS model was developed as a pre-clinical model of depression more than two decades ago (539). Since then, different groups have developed different protocols in an effort to improve its efficiency and tailor it to the particular needs of their research (342,540,541,542,543). The CMS model is mainly designed to produce the characteristic anhedonic-like behavior and general loss of interest towards rewards that is seen in most patients with depression. In this animal model, the behavioral deficits are induced over a period of 3 - 9 weeks by imposing a variety of stressors in a semi-random manner separated by various time intervals (**Figure 10**), which prevents a potential adaptation to the different stressors that is usually seen with continuous application of a single stressor (544).

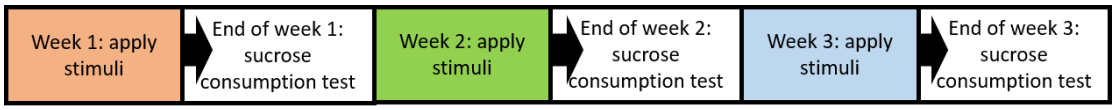

Figure 10. General structure of the chronic mild stress model.
Different stressors (stimuli) are applied weekly and are transiently followed by a test-period of monitored sucrose consumption, to quantify the depressive-like symptoms

The natural preference of rodents to sweetened water is considered to parallel human reward behavior. At the same time, a loss of interest towards reward is one of the clinical characteristics of patients with depression (545). Therefore, the amount of sweetened versus plain water consumed before and after exposure to stressors is used as an index of depressive-like symptoms in the CMS model (546). Different stress stimuli are used between measurements, such as water and food deprivation and cage tilting, which reduces the intake of sweetened water compared to non-stressed control animals (547). Other behaviors, such as, grooming frequency, reduced sexual activity, aggression and reduced locomotion can also be measured as indicators of depressive-like behavior (548,549).

The nature of the protocol allows a combination of a large variety of stressors, with different number/length of intervals and the measurement of different behaviors as a response to rewards

(**Table 6**). Grouped housing, overnight illumination, restraint-stress, day-night cycle disturbance and flashing lights are only a few stressors in a long list of interchangeable stimuli, which are applied to the animal over a certain period, intermitted by a variety of intervals (i.e. 1 – 2 stressors per day with random intervals, 1 stressor per day with 10 intervals *etc*.). Measurement of sucrose consumption during the experiment and preference of sucrose solution over water are examples of recorded behaviors in this model. In addition, the total length of these tests, which varies among CMS studies from 3 weeks to 9 weeks, contributes significantly to the large diversity in the experimental protocols of the CMS model. At present, no study has determined the effect of these variations on the validity or the efficiency of this model. Usually research groups choose a particular combination of stressors and timing based on previous practical experience and/or the particular needs of their experiments. A thorough analysis of these studies is needed to assess the contribution of these variations to the efficiency of the model in order to improve it further.

Table 6. Variability of different protocols exist today in the chronic mild stress model. Variable stressors are applied once or twice a day randomly to induce the depressive-like symptoms, which can be measured by a number of different ways, during a variety of experimental length.

Procedure (weeks)	Animal Species	Stressors applied	Stressor intervals	Main measurement	Example References
3	Wistar rat	• food or water deprivation • cage tilting • intermittent illumination damp sawdust • grouped housing • low-intensity stroboscopic illumination	Stressors applied individually and continuously with 10 - 14 intervals	Amount of consumption of 1% sucrose solution	(550,551,552,553)
3	SD rat	• food or water deprivation • cage titling and wet cage • continuous overnight illumination • grouped housing • low-intensity stroboscopic illumination	1 or 2 stressors per day and randomized intervals		(546,554)
4	SD rat	• food or water deprivation • continuous overnight illumination • cage titling • grouped housing • damp sawdust • stroboscopic illumination	Stressors applied individually and continuously	Test of preference of 1% sucrose solution to plain water	(555)
4	C57BL/6 mouse	• restraint stress • cage titling • grouped housing • white noise • day-night cycle disturbance	Stressor applied individually for 1 hour per day		(556)
4	Lister hooded rat	• food or water deprivation • cage tilting • intermittent or overnight illumination • grouped housing • low-intensity stroboscopic illumination • white noise	First 2 weeks: two stressors per day during daylight Last 2 weeks: two stressors per day during night	Amount of consumption of 0.7% sucrose solution	(557)

Table 6. (Continued) Variability of different protocols exist today in the chronic mild stress model. Variable stressors are applied once or twice a day randomly to induce the depressive-like symptoms, which can be measured by a number of different ways, during a variety of experimental length.

Procedure (weeks)	Animal Species	Stressors	Stressor intervals	Main measurement	Example References
5	Wistar rat	• food or water deprivation • cage titling • intermittent or stroboscopic illumination • soiled cage • paired housing	Stressor applied individually and continuously with 10 – 14 intervals	Amount of consumption of 1% sucrose solution	(558)
5	SD rat	• grouped housing • cage titling and wet cage • food or water deprivation • stroboscopic illumination • white noise • continuous overnight illumination	1 stressor per day, with or without repetition	Amount of consumption of 1% sucrose solution	(559)
6	BALB/c mouse	• grouped housing • noise • damp or remove sawdust • cage changing & tilting • cold water swim • low-intensity stroboscopic illumination • day-night cycle disturbance	1 or 2 stressors per day at different time each day	Measured body weight, coat state and grooming frequency	(560)
6	Wistar rat	• food or water deprivation • restraint stress • forced swimming • flashing light • isolation	One stressor per day at different times each day; repeated at random	Sweet food consumption	(561)

Table 6. (Continued) Variability of different protocols exist today in the chronic mild stress model. Variable stressors are applied once or twice a day randomly to induce the depressive-like symptoms, which can be measured by a number of different ways, during a variety of experimental length.

Procedure (weeks)	Animal Species	Stressors	Stressor intervals	Main measurement	Example References
8	BALB/c mouse	• cage change & tilting • grouped housing • damp or remove sawdust • restraint stress • noise • day-night cycle disturbance	2 stressors per day with randomized combinations and 1 – 2 hrs. interval	Amount of consumption of 1% sucrose solution	(562)
8	Wistar rat	• restraint stress • cage titling • paired housing • nip tail • day-night cycle disturbance	1 stressor per day and each stressor repeated 6 – 7 times across the procedure	Test of preference of 20% sucrose solution to plain water	(563)
9	Wistar rat	• food or water deprivation • cage tilting • intermittent illumination • soiled cage • grouped housing • low intensity stroboscopic illumination	Stressor applied individually and continuously with 10 – 14 intervals	Amount of consumption of 1% sucrose solution	(564)

1.5.3 Animal models of anxiety

1.5.3.1 Light and dark model

The LD model is based on rodents' spontaneous exploration behaviour and their innate aversion to illuminated areas (565). When the animals are subjected to a novel environment, they tend to explore the unfamiliar environment, whereas at the same time try to avoid the novelty. Therefore, the exploratory behaviour in the LD model reflects the interplay of these two conflicting emotions (566). Generally, a two-compartment chamber is used in the LD model but only one compartment is illuminated with white light while the other remains darkened. The size of the illuminated compartment can be either equal to or larger than the unilluminated one (567). In this model, the time that the animal spends in the darkened versus illuminated compartments is used as an index of anxiety-like behaviour (568). Drugs that increase the time in the illuminated compartment are considered as anxiolytics or having anxiolytic-like properties (569). Four additional parameters are also used to reflect the anxiolytic activity of tested drugs: increased transitions between two compartments without increased locomotion, the latency for the first passage from the light compartment to the dark one, the number of transitions between the two compartments and the movement in each compartment (570). Because of the variations in measurements and modifications, discrepant findings are reported across different laboratories (571). Therefore, it is important to analysis multiple parameters carefully based on the specific experimental setup.

1.5.3.2 Fear conditioning and fear extinction model

Fear conditioning

Fear conditioning (FC) or Pavlovian FC is a form of associative learning (572). FC is highly transitional across species and behavioral systems (573). The FC model was intensively used to study psychophysiological processes and brain circuits that identify danger associated with

specific cues or contexts (574). There are two different types of FC, cued conditioning and contextual conditioning (575). In a classical cued FC paradigm, a conditioned stimulus (CS, e.g., a tone) is repeatedly paired and co-terminated with an aversive unconditioned stimulus (US, e.g., a shock), yielding a CS – US association. A conditioned response (CR, e.g., freezing behavior) is produced in response to the paired CS. Animals will exhibit CR to the same CS signal even when they are subjected to different environment (575). While in the contextual FC paradigm, US is presented alone, thus yielding a US-context association. When animals are subjected to the same environment, they will express CR even in absence of the US (576).

Fear extinction

FE is a process used after FC training, where only the CS is presented, leading the CR to decline over repeated presentations. Extinction is not to erase the initial CS-US association, but to create new learning to overcome the association between CS and US (577). Following successful FE, the presentation of CS will only trigger the memory of unpaired CS. Low-level of or absent CR expression in the post extinction test at a later stage, which is also known as extinction recall, indicates the success of FE training (578) (**Figure 11**).

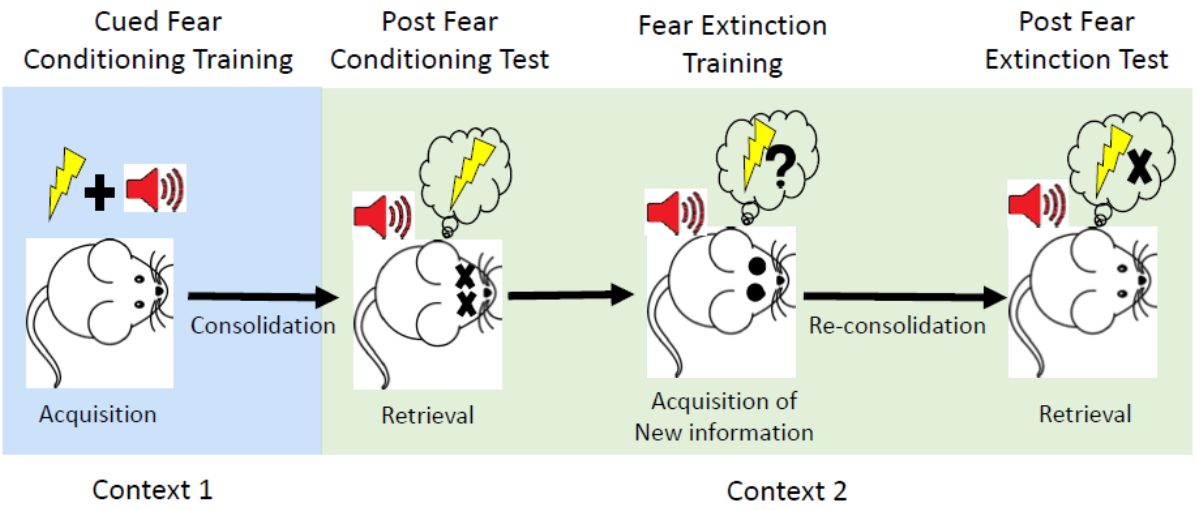

Figure 11. Schematic representation of the classic fear extinction paradigm.
Generally, two contexts are employed. In the first context (blue), rodents receive repeated pairs of CS and US and learn the CS – US association. After a certain period (memory consolidation), the establishment of CS – US association is evaluated in a new context (green). Following the post-FC test (retrieval), animals are given repeated CS in absence of US and learn to de-associate the CS with US (FE training).The post FE test (retrieval) is carried out in the same context that is used in FE training after a certain period (memory re-consolidation) to measure the effect of FE training.

It is notable that fear responses may still reappear spontaneously over time (i.e., spontaneous recovery), by either subjecting animals back to the initial environment or context where they received FC training, or presenting them with the US (579). The interactions between fear conditioning and extinction play an important role to understand the underlying brain circuits that support cognition, stress and anxiety disorders. In addition, reported differential responses to the same stressors that are observed in different strains (580), sex (581) and ages (582) in the FC – FE paradigm can be beneficial to investigate individual differences in fear and anxiety, and are therefore helpful to develop personalized diagnosis and future treatments.

1.5.4 Animal models of nociception

Current animal models of nociception are designed to indirectly monitor pain by measuring their responses to nociceptive stimuli. Methods to stimulate nociceptive responses can be mechanical, chemical, electrical or thermal (167). Measured responses can be mediated by

either peripheral or central nerve systems depending on the type of stimuli used and which part of the body the stimuli are applied to (583,584). Currently, the hot plate (HP) and tail flick (TF) tests are the most commonly used thermal-stimulus based models for acute pain (167).

1.5.4.1 Tail-flick test

The TF test is a nociceptive essay based on the measurement of the latency of the avoidance response to thermal stimulus in rodents (585). It is used to quantify nociception mediated primarily by spinal mechanism. This test has proved particularly sensitive for studying the analgesic properties of pharmacological substances (586) and is performed in two distinct variations. One method induces a nociceptive response by applying radiant heat to a small area of the rodent tail (587), while in the other method the animal's tail is immersed into cold or hot water (588). The TF test with radiant heat is a more simplified version to measure nociception. The application of thermal radiance (e.g., generated by an infrared lamp) to the animal tail provokes a nociceptive response where the tail is vigorously removed from the heat source. A timer records the moment the tail is withdrawn and the heat source is switched off automatically. The latency from the beginning of the measurement to the removal of the tail from the infrared source (called tail flick latency) is interpreted as nociception (585). The time to tail withdrawal can vary based on the intensity of the radiant source (589) and the surface area stimulated by the heat (590). The higher the heat intensity and the smaller the stimulated area, the shorter the TF latency typically is. However, different parts of the rodent tail have different sensitivities towards radiant heat. The distal part of the tail is believed to be the most sensitive, followed by the middle and proximal sections (591).

The TF test is widely used because it does not require sophisticated equipment and is easy to perform. From a pharmacological point of view, it is sensitive to clinically used analgesics (592). For example, it is easy to establish dose- and time-dependencies for morphine-induced

analgesia in rodents at clinical equivalent doses (593). It is also effective to predict the potential analgesic effect of full opioids agonists in humans, even though it seems to be less reliable to detect partial opioid agonists (594). This may be due to the fact that the evaluation of partial agonists is more easily impacted by the experimental setup such as cut-off time and intensity of radiation heat than when detecting full agonists (594). . . However, it is still possible that opioid-based analgesics do not provide substantial effects in this test under certain conditions due to treatment duration and experimental design. Rats, which received morphine for 13 days before exposure to foot shocks, failed to exhibit an antinociceptive effect in the TF test following re-exposure to shocks when compared to non-treated rats (595). This effect could be explained by the release of endogenous opioid ligand β-endorphin in response to a stressful stimulus. This endogenous MOP agonist is cross-tolerant with chronically administrated morphine (595). On the other hand, individual differences of rodents such as the tail temperature may also introduce bias into nociception measurements (596). Therefore, the reliability of this assay relies on several experimental factors, which can contribute to misinterpretation of results. .

1.5.4.2 Hot-plate test

The HP test is designed to measure nociception mediated primarily by a supraspinal mechanism (586). The typical equipment that is used in the HP test includes an open-end cylindrical space with a metallic floor plate (597). The plate is pre-heated to a consistent and pre-defined temperature before placing a rat or mouse into the cylinder. The duration from when the animal initially touches the plate to a defined behaviour such as paw licking or jumping is recorded manually (597). Both paw licking and jumping behaviours are considered as controlled by supraspinal mechanisms (598). Although an early paper claimed that paw licking was only observed following administration of opioid analgesics (167), paw-lick latency has been widely

used in nociception measurements that did not involve opioid administration (597,599,600). .In the HP test, some other behaviours such as sniffing, lifting forepaws and rearing can be observed (601), which increase the difficulties to decide the appropriate endpoint of a measurement. Therefore, compared to the TF test, the HP creates more variability and produces less accurate data. Therefore, to increase the reliability of results, the time until the first observed behaviour, regardless of whether it is paw-licking or jumping, was suggested to be a better measure (602). In addition, it was suggested that learning is involved in the HP test, since shorter pain reaction times were observed after repeated exposure of the test animals to the heated plate (601). It was observed that rats immediately jumped as soon as being placed into the cylinder in subsequent tests, even when the plate was at room temperature (603). It is also worth to notice that a large body area of the test animals (including four paws and the tails) typically has contact to the hot plate, which makes results difficult to interpret since the pain reactions may be a combination of both peripheral and supraspinal pain perception (167). From a pharmacological point of view, the HP test shows good correlative results of drugs that produce antinociceptive effects in rodents and are clinically used for the treatment of pain (604). Especially, the low intensity hot plate test seems to be more sensitive to analgesic drugs (605,606). In addition, this assay is able to evaluate narcotic agonists, antagonists and non-narcotic analgesics (607). Therefore, compared to the TF test, it appears to be more suitable to predict a wider range of compounds with analgesic potential in humans.

In spite of the vulnerability of the TF and HP assays to different factors such as experimental settings and individual differences of animals and their inability to determine pain responses, both assays are still required to detect potential analgesics for human use and play an important role in the area of pain research. Both assays are important tools for the initial screening phase of drug discovery to identify compounds with potential analgesic activity (608) and are also fundamental to understand basic mechanisms of nociception, as well as pain and analgesia.

1.5.5 Animal model of cognition

Cognitive impairment has been reported in patients with depression as one of the depressive symptoms (85). Similarly, changes to cognition have also been demonstrated in stress-based animal models of depression such as in the learned helplessness model (82). However, if a drug is able to improve learning and memory in test animals, potentially false positive results, interpreted as antidepressant effects of the tested drug, can be produced. Therefore, in preclinical models of depression it is essential to also determine the effects of drugs on cognitive function.

In an integrated model of depression and cognition, it is essential to consider the compatibility between two models. For example, it is beneficial to use a model of cognition that does not require external averse stimuli, when integrating it with a stress-based depression model. Based on this reasoning, the novel object recognition (NOR) model, which is purely based on the inquisitiveness of the test animals, has been frequently used. In this model, rodents respond to novelty and can learn to recognize previously presented stimuli (609). The NOR model assesses the ability of test animals to remember a set of objects and whether they can distinguish between a novel and a familiar object. The advantage of the NOR model is that the procedure requires no external motivation, rewards and/or punishment but fully relies on the animals' natural propensity to explore novel objects (610). The NOR task has become a widely used model for investigating recognition memory. With modifications, it was also reported to test working memory, attention and anxiety (611). Yet, it has been used to evaluate the effects of various pharmacological treatments and brain circuits that are involved in cognitive function (611). A typical NOR procedure consists of three phases, including habituation, familiarization and a testing phase. In the habituation phase, each animal is allowed to freely explore the experimental chamber. Then in the following object familiarization phase, the animal is placed back into the same chamber and is allowed to explore two identical objects (A + A). In the

subsequent testing phase, one of the familiar objects is replaced with a novel one (A + B) before the individual exploration time for both objects is recorded. In this paradigm, different methods are used to quantify memory that include discrimination index (DI), index of global habituation and differences in exploration time for both objects (612). This model is based on the assumption that a longer exploring time for the novel object indicates that the familiar object is present in the animals' memory.

The NOR task can be used to study short-term, intermediate-term and long-term memory by manipulating the interval between the object familiarization phase and the testing phase (613). It has to be noted though that a single testing session is more vulnerable in terms of assessing memory function accurately, compared to repeated measurements (614).

1.5.6 Animal model of locomotion

Reduced physical activity is also observed in patients with MDD and long-term physical withdrawal can worsen depressive symptoms (615). To assess drug effects on locomotion in pre-clinical depression models can not only give insights into the interaction between physical activity and depression, but also helps to determine the interference of a potential stimulating effect of a drug on its antidepressant-like effects. The OF model is one of the most frequently used animal models to measure exploratory behavior and general physical activity in both rats and mice. The simplicity of equipment and clear defined behaviors in this test contributes to the popularity of this model (616). Over the past decades, multiple modifications have been applied such as different shapes and sizes of the OF chamber, illumination, floor texture and odors (617). The most basic outcome of this model is "movement", which is represented as the duration of exploration behavior over the total duration of a testing session. It is believed that the longer the exploration is, the more "activity" an animal has (616). However, this outcome can be influenced by many factors such as the attractiveness of testing environment, individual

differences in exploratory behavior and handling. Apart from time spent moving, other parameters, for example, total distance over the testing period have been reported and used as index of locomotion (618). Another benefit of using the OF model is that it is easy to be integrated with other paradigms (e.g., the LD model) to measure the involvement of locomotion and its impact on other behaviors of interest (619). Additionally, physical stimulating, sedative and even anxiolytic effects of a wide range of drugs can be tested in this model (619). Thus, the OF model is able to measure a number of behaviors beyond simple locomotion, such as studying exploratory behavior through measuring the number of rearing events (620). Recently, the OF model has also been used to measure anxiety levels in rodents. The major parameters that are used for this purpose are freezing time and (a cease of all movement except for respiration) time spent staying in central area of testing chamber (621). However, its validity to be used as a model of anxiety is still questionable, because drugs that are clinically effective for the treatment of anxiety showed little effect in the OF model (622).

1.6 Future perspectives

Although a large volume of *in vivo* evidence supports the beneficial use of opioids in depression and anxiety, novel opioids have not been clinically developed as viable alternatives for conventional antidepressants. It is possible that the addictive properties of opioids as well as their tendency to produce tolerance after long-term use are the biggest barriers for the development of opioid-based therapies. Therefore, bifunctional opioids that can potentially avoid opioid receptor-dependent adverse effects while retaining their therapeutic effects, present a promising drug class that should be investigated. At the same time, the complex effects of opioids in the regulation of stress circuits via multiple pathways, requires that the opioid research community generates a deeper insight into opioid pharmacology, especially with regards to the psychological, physiological and neurological activities of opioids. Given

that mitochondria, energy metabolism and oxidative stress are described as downstream targets and effects of opioids (623,624), a general investigation into the use of targeted mitochondrial therapies as antidepressant alternatives also appears warranted. Even more so, since one of the most widely used drug targets for the treatment of depression, MAOA is a mitochondrial outer membrane enzyme. Considering the role of MAO in the onset of various mental disorders (e.g., MDD, anxiety and stress-induced cognitive impairment) and mitochondrial function (e.g., ROS-induced lipid peroxidation of mitochondrial membranes), it appears entirely feasible to develop novel antidepressant strategies by targeting mitochondrial function.

1.7 Aims of the project

This project intended to establish an integrated learned helplessness model in a multiple conditioning system (MCS) to test antidepressant effects of morphine and 2 novel bifunctional opioids synthesized at the University of Tasmania. Validity of the LH model to induce depressive-like symptoms and the effects of novel antidepressants in this model will be systemically assessed by evaluating 4 interference factors: anxiety, pain, cognition and locomotion. Since such a systematic evaluation of the LH model has not been performed before, our results can provide significantly improved guidelines for other researchers to use and adjust the LH model based on their own laboratory setup.

In addition, the relationship between oxidative stress, depression and cognitive function will be investigated further by testing the mitoprotective antioxidant idebenone. The results of this part of the study will give us better understanding of the role of mitochondrial function and oxidative stress in the regulation of cognition under stressful condition.

Overall, this study allows us to explore opioid-based and mitochondrial therapy-based antidepressant alternatives. It is also important that this project can be a cornerstone of

investigating the role of opioidergic system and mitochondrial in depression and its-related comorbidities.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Animals

2.1.1.1 Strains

Sprague Dawley rats (SD, male), 7 weeks, 200 – 310 g at the beginning of experiments	University of Tasmania	Hobart TAS, Australia

2.1.1.2 Animal chow

Barastoc 20% protein rodent diet	102108	Ridley Agriproducts Pty Ltd	Harristown QLD, Australia
Soy-reduced rodents cubes (reformulated), autoclaved	SF06-053	Specialty feeds	Glen Forrest, WA, Australia
Soy-reduced rodents mash, autoclaved	SF06-052	Specialty feeds	Glen Forrest, WA, Australia

2.1.1.3 Animal facility material

Clear plastic ventilated Techniplast cages	1400U	Tecniplast Pty Ltd.	Rydalmere NSW, Australia

2.1.2 Cells

Human hepatocellular carcinoma cell line (HepG2)	85011430	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.3 Software

Leica Application Suite Version 3.4.1	Leica Microsystems	Wetzlar, Germany
Nikon NIS-Elements D413.00 (64-bit)	Nikon Instruments Inc.	Melville NY, USA
SoftMax Pro 4.8	Molecular Devices	Sunnyvale CA, USA
GraphPad Prism (version 6.01)	FigurePad Software, Inc.	La Jolla CA, USA
PhotoShop CS6 (version 13.0, 64-bit)	Adobe Systems	Mountain View CA, USA
Fiji ImageJ	NIH	Bethesda MD, USA
VideoMot2	TSE Systems GmbH	Bad Homburg, Gemany

2.1.4 Instruments

2.1.4.1 Machines

Autoclave	SX-700E	Tomy Tech USA, Inc	Fremont CA, USA
Swing bucket centrifuge	CM-6MT	ELMI Ltd. laboratory equipment	Riga LV, Latvia
Micro centrifuge	5415D	Eppendorf	Hamburg, Germany
Cell Culture Incubator	BBD6220	Heraeus	Hanau, Germany
IKA® MS 1 minishaker	000L001500	H.B.Selby & Co. Pty Ltd	Wilmington NC, USA
PIPETBOY accu	200940	INTEGRA Biosciences	Biebertal, Germany
Magnetic stirring hotplate	MR3001K	Heidolph	Schwabach, Germany
GFL® Water bath (cell culture)	1003	Faust Laborbedarf AG	Schaffhausen, Switzerland
Inverted biological Microscope	INV-100	BEL Engineering	Monza, Italy
Vortex mixer	VM1	Ratex Instruments Pty Ltd.	Boronia VIC, AU
Multiskan™ GO Microplate Spectrophotometer		ThermoFisher Scientific	Scoresby VIC, AU
Fluoroskan Ascent™ FL Microplate Fluorometer and Luminometer		ThermoFisher Scientific	Scoresby VIC, AU
EuroClone Topsafe Class II (Type A2) Biological Safety Cabinets		LAF Technologies Pty Ltd	Bayswater North VIC, AU
Stinger Research Anaesthesia machine		Advanced Anaesthesia Specialists	Gladesville NSW, AU
Peristaltic pump	Peri-Star™ Pro	World Precision Instruments	Sarasota FL, USA
Safe-Tee Fume Cupboard	HC 05/1	Conditionaire International	Marrickville NSW, AU
Raymond Lamb Blockmaster Embedding centre	1347624-W16	Wotol inc.	Lyon, France
Tail flick (TF) unit	37360	Ugo Basile	Comerio VA, Italy
Cold/hot plate unit	35150	Ugo Basile	Comerio VA, Italy
Auto-processor	ASP200	Leica Biosystems	Mount Waverley VIC, AU
Histology water bath	HI1210	Leica Microsystems	Wetzlar, Germany
Microtome	RM2255	Leica Microsystems	Wetzlar, Germany
Coverslipper	CR100	Dako Denmark A/S	Glostrud, Denmark
Light microscope	DM 2500	Leica Biosystems	VIC, AU
Orbital Shaker	PSU-10i	Grant Instruments	Royston, UK
Fluorescence Microscope	BX50	Olympus	Notting Hill VIC, AU

Fluorescence Microscope Excitation Light Source	120Q	Excelitas Technologies Corp.	Wheeling IL, USA
CCD Camera	CoolSNAP HQ2	PhotoMetrics	Tucson AZ, USA
Multiple Conditioning System	256060-L	TSE systems	Bad Homburg vor der Höhe, Germany
Ultrasonic converter		Misonix Incorporated	Farmingdale NY, USA
Ultrasonic cell distributor	XL	Misonix Incorporated	Farmingdale NY, USA
Microplate reader	SpectraMax M2	Molecular Devices	Sunnyvale CA, USA
Centrifuge	Z233 MK-2	HERMLE Labortechnik · Zentrifugen	Wehingen, Germany
Mini Heating Dry Bath Incubator	MD-mini	MS Major Science	Saratoga CA, USA
Vortex	VM79	Selby Scientific Ltd	Mulgrave North VIC, AU
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels, 2-gel	1658005	BioRad	San Diego CA, USA
Mini-PROTEAN® Tetra Handcasting Accessory Kit	1653370	BioRad	San Diego CA, USA
PowerPac™ Basic Power Supply	1645050	BioRad	San Diego CA, USA
Mini Trans-Blot® Module	1703935	BioRad	San Diego CA, USA
Mini-PROTEAN® system Glass Plates	1653308	BioRad	San Diego CA, USA
Mini-PROTEAN® 3 system Glass Plates	1653311	BioRad	San Diego CA, USA
Imager	A600	GE Healthcare Life Sciences	Parramatta NSW, AU

2.1.4.2 Pipettes

P2.5	Q27350C	Eppendorf South Pacific Pty. Ltd.,	NSW, AU
LAMBDA P20	358233382	Corning Incorporated	Corning NY, USA
m100	12561335	Biohit	Helsinki, Finland
m200	12574025	Biohit	Helsinki, Finland
Axygen® Axypet® P1000	358162563	Corning Incorporated	Corning NY, USA
Finnpipette® P10	T19215	ThermoLabsystems	Scoresby VIC, AU
Finnpipette® P50	S46099	ThermoLabsystems	Scoresby VIC, AU
Finnpipette® P300	4510	ThermoLabsystems	Scoresby VIC, AU

2.1.4.3 Miscellaneous

Quick-Chill Box	DS5114-0012	Nalgene	Rochester NY, USA

2.1.5 Recombinant enzymes and antibodies

Luciferase	E1702	Promega Corporation	Alexandria NSW, AU
Anti-MAOA, rabbit, monoclonal	ab126751	Abcam	Melbourne VIC, AU
Anti-nitrotyrosine, rabbit, polyclonal	A-21285	Invitrogen	Scoresby VIC, AU
Negative control rabbit immunoglobulin fraction (normal)	X0903	Biocompare	South San Francisco, USA
Goat anti-rabbit IgG H&L (Alexa Fluor® 594)	ab150084	Abcam	Melbourne VIC, AU
Goat pAb to Rb IgG (HRP)	ab97051	Abcam	Melbourne VIC, AU
Biotinlated anti-rabbit IgG, from Vectastain® ABC kit	PK-4001	Biocompare	South San Francisco, USA

2.1.6 Media and cell culture supplements

2.1.6.1 Cell culture media

Dulbecco's Modified Eagle's Medium (DMEM) – low glucose	D5523	Sigma-Aldrich	Castle Hill, NSW, AU
DMEM – without glucose	D5030	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.6.2 Serum

Fetal Bovine Serum, sterile (FBS, cell culture)	SFBS-F	Bovogen Biologicals Pty Ltd.	Kellor East Vic, AU
Bovine Albumin, Fraction V, Protease-free, 96.99% (histology)	A-3294	Sigma-Aldrich	Castle Hill, NSW, AU
Horse Serum	26050	Gibco	Melbourne VIC, AU

2.1.6.3 Cell culture chemicals

Penicillin Streptomycin	15140-163	Gibco	Melbourne VIC, AU

0.25% Trypsin-EDTA (1x)	25200-056	Gibco	Melbourne VIC, AU
Ethylenediaminetetraacetic acid (EDTA) solution	03690	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.7 Chemicals

2.1.7.1 Drugs

1001	10mM (cell culture), 12.5mg/mL (*in vivo*)	University of Tasmania	TAS, AU
1003	10mM (cell culture), 12.5mg/mL (*in vivo*)	University of Tasmania	TAS, AU
Morphine sulphate (cell culture)	1mM	Proprietor Provet Victoria Pty Ltd	TAS, AU
Morphine sulphate solution, 30mg/mL (*in vivo*)	2.5 and 5mg/mL	Hospira AU Pty Ltd.	TAS, AU
Imipramine hydrochloride	25 and 50mg/mL	Abcam plc	Cambridge, UK
Idebenone	200mg/kg	Santhera Pharmaceuticals	Switzerland

2.1.7.2 Anesthetics

ISOTHESIA® inhalation Anaesthetic 100% Isoflurane	9008931	ProVet Tasmania	Tasmania
Pentobarbital sodium	52356V1	Virbac Animal Health	Milperra NSW, AU

2.1.7.3 Chemicals

Carboxymethylcellulose (CMC), sodium salt		C9481	Sigma-Aldrich	Castle Hill, NSW, AU
EDTA	0.5M	1610729	BioRad Laboratories, Inc.	San Diego CA, USA
L-glutamine		G5792	Sigma-Aldrich	Castle Hill, NSW, AU
Citric acid		C0759	Sigma-Aldrich	Castle Hill, NSW, AU
Sodium bicarbonate		S5761	Sigma-Aldrich	Castle Hill, NSW, AU
Phosphate buffered saline (PBS) tablets		18912014	Life technologies	Mulgrave VIC, AU
Sodium dodecyl sulphate (SDS)	10%(w/v)	1610361	BioRad Laboratories, Inc.	San Diego CA, USA
30% Acrylaminde/Bis Solution, 29:1		1610156	BioRad Laboratories, Inc.	San Diego CA, USA
Phenylmethanesulfonyl fluoride (PMSF)	100mM	78830	Sigma-Aldrich	Castle Hill,NSW, AU
DL-Dithiothreitol (DTT)	1M	43815	Sigma-Aldrich	Castle Hill, NSW, AU

Glycerol		G7893	Sigma-Aldrich	Castle Hill, NSW, AU
Magnesium chloride ($MgCl_2$)		M4880	Sigma-Aldrich	Castle Hill, NSW, AU
HEPES		H4034	Sigma-Aldrich	Castle Hill, NSW, AU
D-(+)-Galactose		G5388	Sigma-Aldrich	Castle Hill, NSW, AU
Bromphenol blue		114391	Sigma-Aldrich	Castle Hill, NSW, AU
Sucrose		S0389	Sigma-Aldrich	Castle Hill, NSW, AU
3,3′-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride		D3939	Sigma-Aldrich	Castle Hill, NSW, AU
Ammonium persulfate		A3678	Sigma-Aldrich	Castle Hill, NSW, AU
Adenosine 5′-triphosphate disodium salt hydrate (ATP)		A2383	Sigma-Aldrich	Castle Hill, NSW, AU
N,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED)		T9281	Sigma-Aldrich	Castle Hill, NSW, AU
Dimethyl sulfoxide (DMSO)		D4540	Sigma-Aldrich	Castle Hill, NSW, AU
Paraformaldehyde (PFA)		158127	Sigma-Aldrich	Castle Hill, NSW, AU
Sodium fluoride		201154	Sigma-Aldrich	Castle Hill, NSW, AU
NP-40			Sigma-Aldrich	Castle Hill, NSW, AU
Sodium deoxycholate		D6750	Sigma-Aldrich	Castle Hill, NSW, AU
Sodium orthovanadate	200mM	S6508	Sigma-Aldrich	Castle Hill, NSW, AU
Sodium chloride (NaCl)		27810.295	VWR Chemicals	Tingalpa QLD, AU
Sodium hydroxide (NaOH), pellets		221465	Sigma-Aldrich	Castle Hill, NSW, AU
Glycine		G8898	Sigma-Aldrich	Castle Hill, NSW, AU
D-Luciferin		L9594	Sigma-Aldrich	Castle Hill, NSW, AU
Dako Fluorescent Mounting Medium		S3023	Dako North America, Inc.	Carpinteria CA, USA
Sodium phosphate dibasic		S5136	Sigma-Aldrich	Castle Hill, NSW, AU
Potassium phosphate monobasic		P5655	Sigma-Aldrich	Castle Hill, NSW, AU
Potassium chloride		P5405	Sigma-Aldrich	Castle Hill, NSW, AU
Sodium phosphate dibasic		S5136	Sigma-Aldrich	Castle Hill, NSW, AU
Trizma® base		T1503	Sigma-Aldrich	Castle Hill, NSW, AU
Trizma® hydrochloride		T5941	Sigma-Aldrich	Castle Hill, NSW, AU
Triton® X-100	20%(v/v)	T8532	Sigma-Aldrich	Castle Hill, NSW, AU
Tween 20	20%(v/v)	1706531	BioRad Laboratories, Inc.	San Diego CA, USA
Dako Hematoxylin		CS700	Dako Denmark A/S	Glostrup, Denmark
Hydrogen peroxide solution, 30wt % in H_2O		216763	Sigma-Aldrich	Castle Hill, NSW, AU
PageRuler™ prestained protein ladder		26616	Life technologies	Scoresby VIC, AU

Enhanced chemiluminescence western blotting reagent 1	RPN2109D1	Amersham Bioscience UK Pty Ltd	Buckinghamshire, UK
Enhanced chemiluminescence western blotting reagent 2	RPN2109D2	Amersham Bioscience UK Pty Ltd	Buckinghamshire, UK

2.1.7.4 Fluorescent dyes

BODIPY-C_{11} 581/591 (4,4-difluoro-5-(4-phenyl-1,3- butadienyl)-4-bora-3a,4a-diaza-s-indacene- 3-undecanoic acid)	D3861	Invitrogen	Carlsbad CA, USA
CM-H_2DCFDA (5-(and-6)-chloromethyl- 2',7'-dichlorodihydrofluorescein diacetate, acetyl ester)	C6827	Invitrogen	Carlsbad CA, USA
DAPI (4′,6-diamidino-2-phenylindole dihydrochloride)	D9542	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.7.5 Kits

DC Protein Assay kit	5000116	BioRad	San Diego CA, USA
Vectastain® Elite ABC-Peroxidase Staining Kit	PK6100	Biocompare	South San Francisco, USA

2.1.8 Consumables

2.1.8.1 Cell culture flasks

25cm^2 (T-25) Cell Culture Flask	430639	Sigma-Aldrich	Castle Hill, NSW, AU
75cm^2 (T-75) Cell Culture Flask	430641	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.8.2 Microplates

96-well plate, sterile, flat bottom, with lid, clear	3596	Sigma-Aldrich	Castle Hill, NSW, AU
96-well plate, sterile, flat bottom, with lid, black	3916	Sigma-Aldrich	Castle Hill, NSW, AU
96-well plate, non-sterile round bottom, no lid, white	3789	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.8.4 Filters

33mm Syringe Driven Filter Unit, 0.22 µm, sterile	SLGP033RS	Adelab Scientific	Thebarton, South AU
500mL Bottle Top Filter, 0.22 µm, PES, sterile	431118	Sigma-Aldrich	Castle Hill, NSW, AU

2.1.8.5 Pipette tips

0.1-10µL	18812000	Corning Incorporated	Corning NY, USA
200µL	LAC1702	VWR LabAdvantage	Tingalpa QLD, AU
300µL	LAC30804	VWR LabAdvantage	Tingalpa QLD, AU
100 -1250µL	53508-914	VWR LabAdvantage	Tingalpa QLD, AU

2.1.8.6 Tubes

2.0mL Cryotubes	430488	Sigma-Aldrich	Castle Hill, NSW, AU
Corning 15mL Centrifuge Tubes	430791	Sigma-Aldrich	Castle Hill, NSW, AU
Corning 15mL Centrifuge Tubes	352070	Sigma-Aldrich	Castle Hill, NSW, AU
PCR tubes, 0.2mL, with attached caps	732-2572	VWR LabAdvantage	Tingalpa QLD, AU
Eppendorf tube 1.5mL	759987	Livingstone International	Rosebery NSW, AU
Eppendorf tube 0.5mL	EP0030121023	Livingstone International	Rosebery NSW, AU

2.1.8.7 Microscope slides and coverslips

IHC microscope slides	K8020	Dako Agilent Pathology Solutions	Mulgrave VIC, AU
Cover glass (24×50mm)	CS704	Dako Agilent Pathology Solutions	Mulgrave VIC, AU

2.1.8.8 Syringes and needles

Ultra-Fine Insulin syringe	326103	Becton Dickinson	North Ryde NSW, AU

1mL disposable syringe	352070	Livingstone International	Rosebery NSW, AU
26G×3/8”	300300	Becton Dickinson	North Ryde NSW, AU

2.1.8.9 Miscellaneous

Tissue-Tek® III Stacked Cassette	4154	Sakura	Alphen aan den Rijn Netherlands
Protein nitrocellulose membrane, 0.2µm	10600001	Whatman	Parramatta NSW, AU

2.2 *In vitro* studies

2.2.1 Cell culture and maintenance

2.2.1.1 Routine culture of cells

HepG2 cells were cultivated under standard conditions (37 °C, 5 % CO_2 and 95 % relative humidity [rH]) in DMEM containing low glucose (1 g/L) and L-glutamine. The media was supplemented 1 % (v/v) antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), 10 % (v / v) FBS and bicarbonate (3.7 g/L).

2.2.1.2 Passaging of cells

The routine passaging of HepG2 cells was performed under sterile conditions in a class II biosafety cabinet every 3 - 4 days when the cell reached approximately 80 % confluency. The cells were seeded in T-25 flasks with 10 mL of low glucose DMEM medium at a density of 2 million cells / flask. Confluent cells were first washed once with 10 mL PBS without Ca^{2+} and Mg^{2+}, followed by a brief wash with 1 mL of 1 mg/mL EDTA solution for 15 s. Then the cells were incubated with 1 mL of 0.05 % Trypsin (0.5 g/l) / EDTA (0.2 g/l) solution for 5 min until cells detached from the growth surface. Detached cells were resuspended in culture medium at 1:9 (trypsin:culture media) dilution and then counted using a haemocytometer. Once

thoroughly suspended, cells were seeded at a density of $8x10^4$ cells / cm^2 in either T-25 or T-75 flask.

2.2.1.3 Freezing of cells

To freeze cells for longer-term storage or as back up, cell suspensions were prepared (detailed protocol see 2.2.1.2). Cells were collected by centrifugation (500 x g for 5 min) using a swing bucket centrifuge at room temperature. The pellet was resuspended in 1 mL of cryo medium (low glucose DMEM, 20 % FBS, 10 % DMSO). The cells were cooled down at a constant rate (1 degree / min) in a -80 °C freezer for 24 h using a Quick-Chill unit, followed by transfer into the liquid nitrogen gas phase for long-term storage.

2.2.1.4 Thawing of Cells

After removing the cryotubes from liquid nitrogen storage, cells were rapidly thawed in a water bath at 37 °C. The cell suspension was immediately transferred into a T-25 flask with 10mL pre-warmed culture medium. The cells were cultivated overnight in CO_2 incubator under standard conditions. To remove residual DMSO, the culture medium was replaced with fresh medium once the cells were fully attached to the bottom of the flask. At least three standard passage cycles were conducted before using the cells for any experiments (detailed protocol see 2.2.1.2).

2.2.2 Assays

2.2.2.1 ATP assay

Glucose-free DMEM medium (1 g/L D-(+)-Galactose, 3.7 g/L bicarbonate, 0.584 mg/L L-glutamine, 1 % (v/v) of 100 units/mL penicillin and 100 µg/mL streptomycin, 2 % (v/v) FBS) was used for the ATP assay. Cells were seeded in this galactose-DMEM medium at 20,000

cells/well in 96-well culture plates in a volume of 100 µL/well. After 24 h, the culture medium was replaced with 100 µL fresh galactose-DMEM medium with or without test compounds. After the treatment period, cells were washed twice with 100 µL room temperature (RT) PBS and lysed in 40 µL of lysis solution (4 mM EDTA, 0.2 % Triton X-100) for 10 min on a shaker at 200 rpm at RT. For measuring ATP content in the lysate, 10 µL of lysate was transferred into a white 96 well culture plate before 100 µL of ATP measurement buffer (25 mM HEPES pH 7.25, 300 µM D-luciferin, 5 µg/mL firefly luciferase, 75 µM DTT, 6.25 mM $MgCl_2$, 625 µM EDTA and 1 mg/mL bovine serum albumin (BSA)) was added. ATP measurement buffer was stored as separate substrate (25 mM HEPES pH 7.25, 600 µM D-luciferin, 75 µM DTT, 6.25 mM $MgCl_2$, 625 µM EDTA and 1 mg/mL BSA) and enzyme solutions (25 mM HEPES pH 7.25, 10 µg/mL firefly luciferase, 75 µM DTT, 6.25 mM $MgCl_2$, 625 µM EDTA and 1 mg/mL BSA) at -80 °C. Luminescence was quantified immediately using a Fluoroskan Ascent™ FL plate reader. ATP levels for each individual experiment were averaged from six replicate wells/sample for both the control and each drug concentration. ATP levels were calculated using a standard curve (0 - 10 µM) for each experiment before values were standardised to protein levels determined by BCA assay (protein assay see 2.2.2.2). The result of each treatment was calculated as ATP content per mg protein and represented as percentage of untreated control. The measurement of cellular ATP levels was performed as three independent experiments and presented using the average values (+/- SD).

The effects of morphine and the novel bifunctional opioids 1001 and 1003 on cellular ATP levels were tested in the ATP assay. Morphine sulphate stock (1 mM in PBS) was stored at 4 ºC and was further diluted to 10 µM using galactose-containing DMEM medium immediately before use. Each well treated with morphine contained 10 % PBS (v/v). Similarly, 1001 and 1003 stocks (10 mM in DMSO, aliquoted and stored at -20 ºC until use) were diluted in galactose-containing DMEM medium to a final concentration of 10 µM. This resulted in a final

concentration of 0.1 % DMSO (v/v) per well, which on its own did not affect the viability of the cells (unpublished observation).

2.2.2.2 Protein assay

This assay is based on the reaction of protein with alkaline copper tartrate (cuprous ions). Initially, aromatic amino acids are oxidized by copper (II), which then reacts as copper (I) with Folin reagent, which produces a characteristic blue colour with a maximum absorption at 750 nm. Protein levels of cell lysates were determined using the BioRad DC protein assay kit according to the manufacturer's instruction with small variations. Briefly, 10 µL of cell lysate or protein standards (BSA: 0 - 2 mg/mL) were added into individual wells of a clear 96 well plate in triplicate, followed by addition of 25 µL reagent A', which is a mixture of reagent S and reagent A at a ratio of 1:50 respectively. Subsequently, 200 µL of reagent B was added to each well. After a 15 min incubation at RT, absorption at 750 nm was measured using a Multiskan™ GO Microplate Spectrophotometer. Protein concentrations were calculated using a BSA standard curve (0 - 2 mg/mL). The measurement of protein content in lysates was presented using average values (+/- SD) from three independent experiments.

2.2.2.3 Reactive oxygen species assay

2.2.2.3.1 Measurement of cellular ROS levles

Measurement of cellular ROS levels using an indicator dye can act as an indirect measurement of oxidative stress levels. A commonly used fluorescent dye that measures hydroxyl, peroxyl and other oxygen radicals is CM-H_2DCFDA. After passively diffusing into the cells, its acetate groups are de-acetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescin (DCF). DCF is highly fluorescent and can be detected with maximum excitation and emission peaks of 495 nm and 529 nm respectively.

The presence of cellular ROS is measured as an increase in fluorescence signal. A CM-H_2DCFDA stock solution (1 mM in DMSO) was prepared, aliquoted and stored at -20 ºC until use. Upon usage, the dye was further diluted to 10 µM using galactose-containing DMEM media.

To measure cellular ROS levels, HepG2 cells were seeded in galactose-containing DMEM medium at a density of 20,000 cells/well in black 96-well culture plates. After 24 h, the wells were washed with 100 µL of PBS. Cells were then loaded with 50 µL of 10 µM CM-H_2DCFDA solution for 30 min, followed by washing with 100 µL PBS to remove excess dye. The cells were subsequently treated with 1 mM tyramine and fluorescence was measured every 15 min over a 75 min period using a Fluoroskan Ascent™ FL plate reader at Ex/Em 495/529 nm.

2.2.2.3.2 Measurement of lipid peroxidation

A fluorescent probe for the ratiometric detection of lipid peroxidation in cellular membranes is BODIPY-C_{11} 581/591. It is a lipophilic dye that enters the lipid bilayer membrane due to its C_{11} carbon side chain. It is oxidized by oxygen radicals or lipid peroxides inside the membrane, which results in a fluorescence emission shift from 590 nm (red) to 510 nm (green). The green (oxidized form) and red (non-oxidized form) fluorescence of BODIPY-C_{11} 581/591 was quantified using a Fluoroskan Ascent™ FL plate reader (Ex/Em 460/535 nm and 485/600 nm). After solubilizing in DMSO, the dye was stored as single use aliquots. The stock solution (1 mM in DMSO) was aliquoted into 10 µL portions and kept frozen at -20 ºC. Upon usage, one aliquot of the BODIPY-C_{11} 581/591 stock was further diluted to a final concentration of 10 µM using galactose-containing DMEM medium. To measure cellular lipid peroxidation, HepG2 cells were seeded in galactose DMEM medium at a density of 20,000 cells/well in black 96-well culture plates. After 24 h, the wells were washed with 100 µL of PBS. Cells were then loaded with 50 µL of 10 µM BODIPY-C_{11} 581/591 solution for 30 min, followed by a wash

with 100 µL PBS to remove excess dye. The cells were treated with 1 mM tyramine and fluorescence was measured every 15 min over a 75 min period using a Fluoroskan Ascent™ FL plate reader.

2.2.2.3.3 Determination of tyramine concentrations

To determine the optimal working concentration of tyramine, a range of drug concentrations (0, 0.1, 1, 10, 100 and 1000 µM) were tested following the method described in 2.2.2.3.1. HepG2 cells were incubated with different concentrations of tyramine for 60 min and fluorescence was measured using a Fluoroskan Ascent™ FL plate reader at Ex/Em 495/529 nm. The data was averaged from six replicate wells/sample and was performed as three independent experiments. Figures show average values from three independent experiments (+/- SD).

2.3 *In vivo* studies

2.3.1 Drug preparation

Morphine sulphate stock solution (30 mg/mL) was diluted to 2.5 mg/mL and 5 mg/mL stocks in saline (0.90 % w/v of NaCl in MilliQ water, pH 7.4, sterile) and stored (RT) in a locked cabinet according to the storage rules for schedule 8 drugs. The tricyclic antidepressant imipramine hydrochloride was dissolved in sterile saline as 25 mg/mL and 50 mg/mL stock solutions, which were aliquoted and stored at -20 ºC until use.

Injection solutions of 1001 and 1003 were prepared under sterile conditions. Drug powder (10 mg) was dissolved in 200 µL of 20 % DMSO in saline, pH 7.4 using a vortex mixer (VM1) at maximum speed. The resulting solution was further diluted with a total of 600 µL saline, which was added slowly as 4 equal amounts to avoid precipitation. The final 1001- and 1003-solutions (12.5 mg/mL in 2.5 % v/v DMSO/saline) were aliquoted and stored at -20 ºC until use.

Idebenone powder was suspended in 0.5 % (w/v) carboxy methylcellulose (CMC) / MilliQ water solution to form a homogeneous suspension by overnight stirring at 4 ºC, before being mixed with soy-reduced autoclaved food powder containing 5 % sucrose. The resulting mixture was aliquoted into 6.3 g portions on balancing trays to provide 200 mg/kg per administration (average daily food consumption amount of 7-week SD rats is 15 - 25g (48)) and stored at -20 ºC. Similarly, same amount of 0.5 % (w/v) CMC solution without drug was prepared for vehicle pellets (6.3 g each).

2.3.2 Drug administration regimes

2.3.2.1 Opioids

The daily doses of morphine (5 mg/kg/day) and imipramine (50 mg/kg/day) were injected subcutaneously followed previously described injection regime with modifications (525,625) Briefly, the first injection at full dose was administrated 1 hour after the 2nd induction session

of the LH model on day 2 (See 2.3.4.3 for detailed protocol of the LH model). After 48 h, drugs were injected twice a day at half-dose per injection. Animals received the initial half-dose 30 min before the 1st LH test and the second half dose 4 h after the LH test on day 4. This routine was repeated on day 5. The final injection (full dose) was administered 1 h before the last LH test on day 6 (**Figure 12**). The selection of morphine dose in my study was based on a previous pharmacokinetic study, where stable plasma concentrations of total morphine were observed up to 6 hours after systemic administration of 5 mg/kg morphine in male SD rats (626). In addition, one injection of 2 mg/kg and 5 mg/kg morphine (s.c,) reached peak brain concentrations of 0.26 µg/g 1 h and 0.179 µg/g 30 mi n after injection in rats and gave stable brain levels for 4 h at the lower dose (627). Since the LH testing procedure in present study lasted for approximately 30 mins, a 2.5 mg/kg/injection was able to maintain a stable brain concentration of morphine throughout the LH testing session. In addition, the purpose of the morphine injection regimen was to avoid the development of morphine tolerance. Repeated injections of 2.5 mg/kg twice a day (tid) was shown to produce tolerance from day 3 onwards in the TF assay, while this was not observed following doses of 5 mg/kg (tid) or higher in male SD rats (628). Therefore, 2.5 mg/kg (tid) was only used for the first two days before the dosing was changed to single dose injections of 5 mg/kg/day to avoid tolerance as described (628).

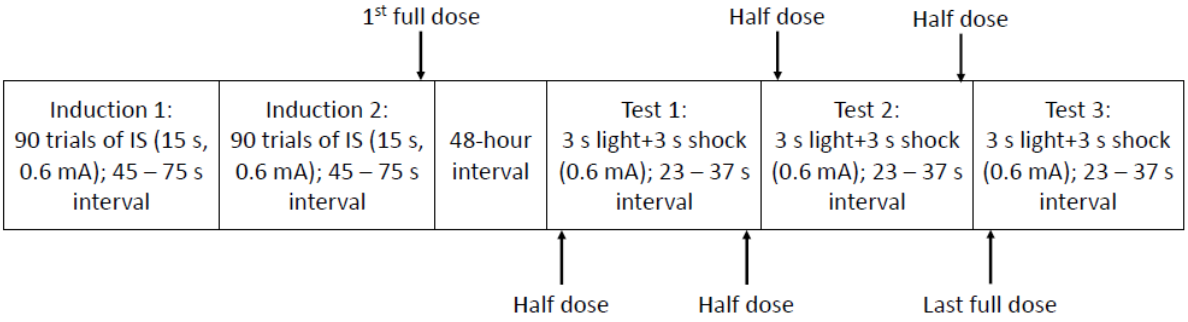

Figure 12. Injection regime of opioids and imipramine.
Male SD rats received 6 injection (s.c.) in total. Both the first and last injection were given as a full dose on days 2 and 6, while the dose was split into two half doses per day, 30 min before and 4 h after the LH tests respectively on days 4 and 5.

Because the pharmacokinetics of UTA1001 and UTA1003 have not been reported or tested so far, both bifunctional opioids were administrated following the morphine injection regimen to compare the behavioural effects between the novel opioids and morphine.

2.3.2.2 Idebenone

Idebenone was used at a wide range of doses (10 – 2000 mg/kg per administration) in previous studies and no toxicity was reported (384,391). Among those, 200 mg/kg has demonstrated neuroprotection of retina in animal study (629). Due to the similarity between the brain retina barrier and the brain blood barrier (630), 200 mg/kg/day was selected for my experiment to ensure sufficient amount of idebenone would penetrate into the brain. Oral administration of idebenone with food mash has also been used (629), thus idebenone (200 mg/kg/day) was administrated through the food mash pellets between 17:30 and 18:00 daily according to two regimes. In one regime, called pre-learning treatment, idebenone was given for 7 days in total, starting from 4 days before the NOR procedure (see 2.3.8.3 for detailed protocol of the NOR model) until day 3. In the other regime, called post-learning treatment, the drug was administrated from days 4 to 6 (**Figure 13**). The drug / food mash pellets were fully thawed at RT before placing them into the cage of single housed animals. During the experiment, animals had always access to normal food pellets *ad libitum*. Typically, animals preferred the sucrose-laced food and preferentially ate the drug-containing portions before feeding on the standard food pellets. This method allowed exact monitoring of idebenone intake in a way that did not affect their behavior.

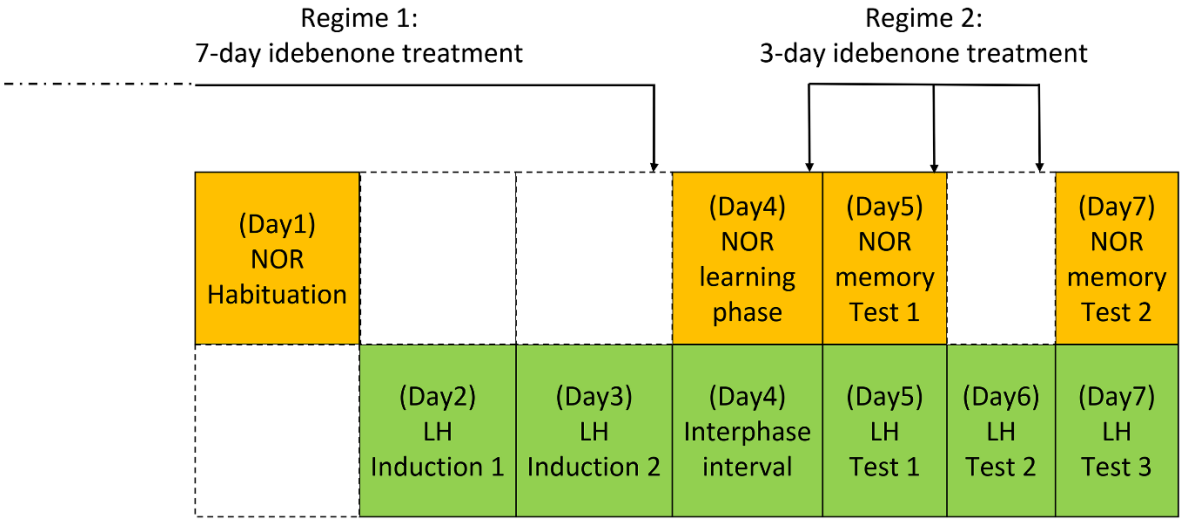

Figure 13. Administration regime of idebenone.
Idebenone (200 mg/kg/day) was given daily for either 7 days before the learning phase (pre-learning) or for 3 days after the learning phase (post-learning) via drug-laced food portions.

2.3.3 Animal handling and maintenance

All procedures were carried out in accordance with the Australian Code for the Care and Use of Animals for the Scientific Purposes and incorporated the 3R principles of Replacement, Reduction and Refinement. All use of laboratory animals was approved by The University of Tasmania Animal Ethics Committee (AEC, #A0013857). All techniques that involved animals were competency assessed by the University veterinarian before used for this project. Male SD rats that were used in this project were siblings, which minimised the influence of inter-individual differences on testing the pharmacological effects of the drugs of interest (631). All 6-week-old animals were delivered weekly to the rat Bio-Bubble pavilion of the Medical Science Precinct (MSP, University of Tasmania) animal facility, at a quantity of 3 – 4 rats per week until the end of the experiments. All rats were housed under a 12 hour light/dark circle (7:00 – 19:00) at 22 ± 0.5 ºC upon arrival. In the LH paradigm, negative emotional states can be induced by the use of electric shocks (632), which can be communicated though ultrasonic vocalisation by rats within a social group (633). Therefore, all animals in my study were single housed to reduce direct communication of negative emotional states. In addition, in the

idebenone experiment, it was necessary to house the animals individually to ensure accurate and reproducible dosing of idebenone to each rat through food/drug pellets. In addition, the behaviour of rats was closely monitored at least 30 mins before and after the experiments under the supervision of the animal technician and the university vet. Those scored behaviours included fur/coating condition, level of aggressiveness during handling, routine activities/locomotion in the cage, eating behaviour, grooming, various signs for different levels of pain and unexpected injuries. Rats were used for experiments only if no abnormalities were observed. These measures were implemented to minimise the bias due to housing conditions. Rats were allowed to adjust the light-dark cycle and other environmental conditions for 1 week before being used in experiments. Clear plastic ventilated cages were used for housing, which offered standard housing conditions that also contained a sleeping house, bedding and a plastic toy. Rats were able to access standard Barastoc 20 % protein rodent diet and water *ad libitum*.

2.3.4 Randomisation and allocation

All animals that were delivered weekly from the university animal breeding facility were randomly allocated into different arms of the experiments as previously described (634) with the following exception. During the period of establishing the effects of morphine on animal behaviour, 6-week-old rats arrived at the MSP animal facility that significantly varied in body weight. In batches 1 to 5, rats arrived at the experimental facility with body weights between 258 to 356 g. Since neither morphine nor imipramine were available during those weeks due to import problems into Australia outside my control, animals were allocated into the NS, IS and ES groups. By the end of week 5, the experimental procedures of the NS and IS groups were fully completed, but not the ES group. From week 6 to 11, rats arrived the MSP animal facility in weekly batches with significantly lower body weights between 178 to 258 g. Those smaller animals were used in the treatment groups (morphine and imipramine) and the ES

group. Because of the differences in rat body weights upon arrival from the breeding facility at different weeks, at the end of the morphine-imipramine experiment, a discrepancy in average body weights between the NS, IS and two treatment groups was seen. Furthermore, due to the unavailability of testing compounds during the initial 5 weeks, it was impossible to perform controls and the treatment groups simultaneously between weeks 1 to 5. Detailed information have been listed in the Appendix (A4) to illustrate animal allocations and body weights for each animal and treatment group (**Table 10 and 11**).

In contrast, for the experiment that aimed to establish idebenone effects, all animals supplied weekly were of similar weight. Hence, rats were randomly allocated into both control and treatment groups throughout the experiment following a complete randomization method (634). For the experiment that aimed to establish the effects of the novel opioids, no additional contemporaneous controls were employed due to the robustness of the LH model. Therefore, weekly delivered rats were only allocated into either the 1001 or 1003 treatment groups.

2.3.5 The multiple conditioning system

The MCS provides a platform for a range of diverse tests and consists of paradigms for anxiety, locomotion, cognition, addiction and depression. It consists of a sound-attenuated box with a light barrier frame that contains three infrared light-beam systems with sensors. Each infrared beam system includes 31 light barriers with 14 mm distance between each barrier. Two of the systems were detecting movement in horizontal X-Y plane, allowing to measure the moving distance, the transfer counts from one compartment to the other in a two-compartment chamber and the number and duration of freezing episodes. The last sensor system was assembled on the top of the Y system and was designed to detect the movement in the vertical (Z) plane such as the number of rearings (XYZ bipedal standing), jumps (Z only), climbs (XYZ bipedal) and leaning/upward movements against chamber walls. The light-barrier frame had a push-pull

style and supported different experimental chambers. In addition, two additional observation LED lights were installed outside of the MCS box to indicate individual foot shock events.

A grid floor for delivering foot shocks was placed underneath the experimental chambers. It measured 30 × 30 cm and was composed of 25 stainless steel rods (ø = 6 mm) that were 18 mm apart from each other. On the ceiling of the MCS housing box, 4 lights (10 W) were assembled as a house light, which were covered with removable red Plexiglas boards, to provide a maximum of 15 Lux red-light and 250 Lux white-light illumination. Two additional lamps were installed between each two house lights that were covered by a sliding blue board to provide stimulus illumination. The intensities of both housing lights and stimulus lights were controlled by the central control unit. A camera was installed 30 cm above the X-Y infrared frame to take the real-time recordings. A speaker was installed on the side of the camera that delivered tone and white noise. The activities of animals in the dark experimental chamber were observed through an infrared-light/camera setup (**Figure 14**). The experimental data was automatically recorded by the associated control software packages (MCS Two-CP PA-PP-LA, MCS ActiMot-OF and MCS Fear Conditioning) for the different paradigms.

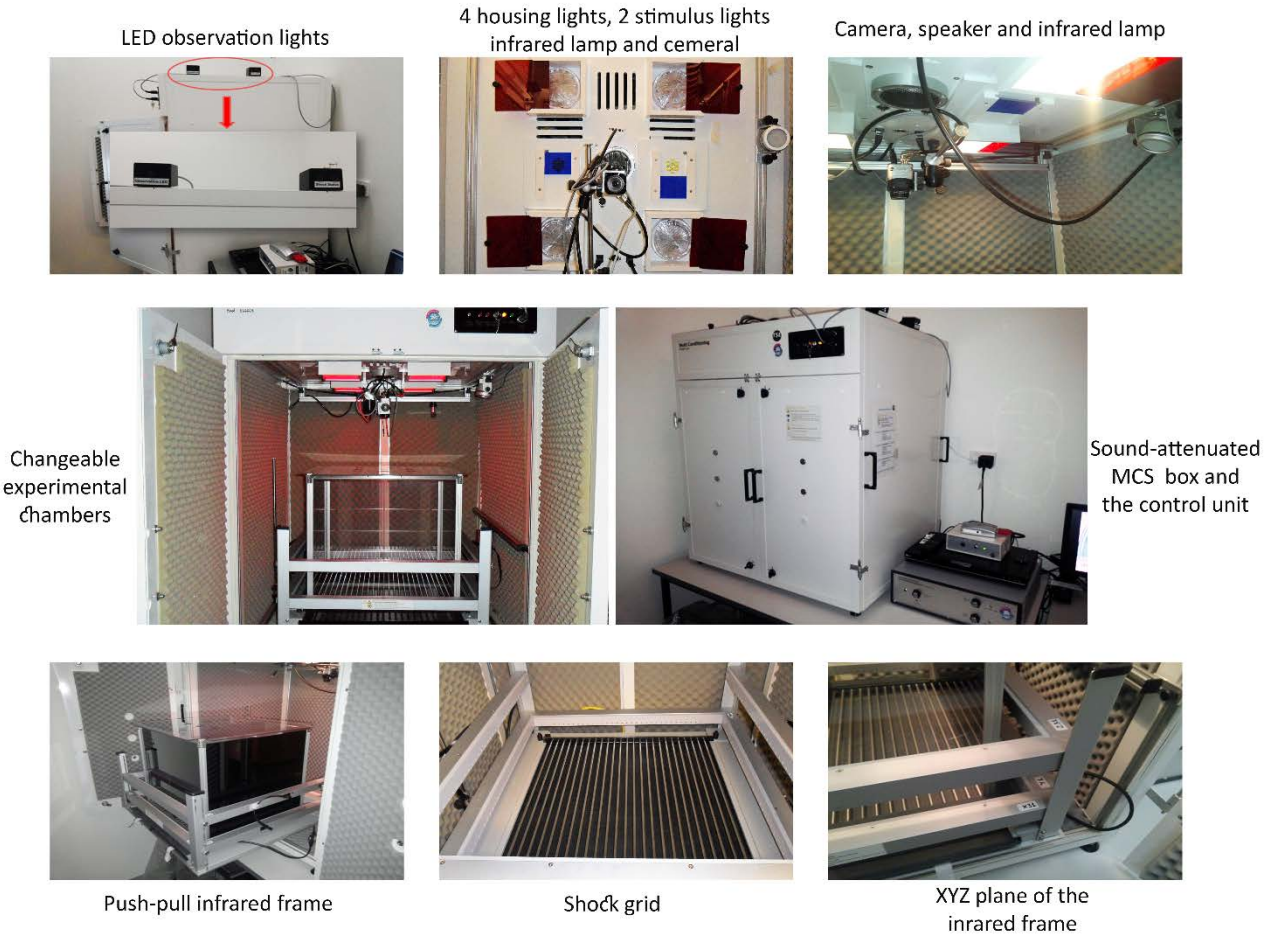

Figure 14. Major components of the multiple conditioning system.

2.3.6 The learned helplessness model

2.3.6.1 Apparatus

The LH procedure is performed in a two-compartment conditioning chamber (458 × 458 × 403 mm) in the MCS system, which is divided by a removable black Plexiglas board with opened arch way in the middle. This chamber is composed of 2 black walls on the side and another 2 clear walls in the front and rear respectively. It has no fixed bottom, which allows the passage of electric foot shocks from the stainless steel grid. A transparent lid is placed on the top to stop the escape of animals during shock trials (**Figure 15**).

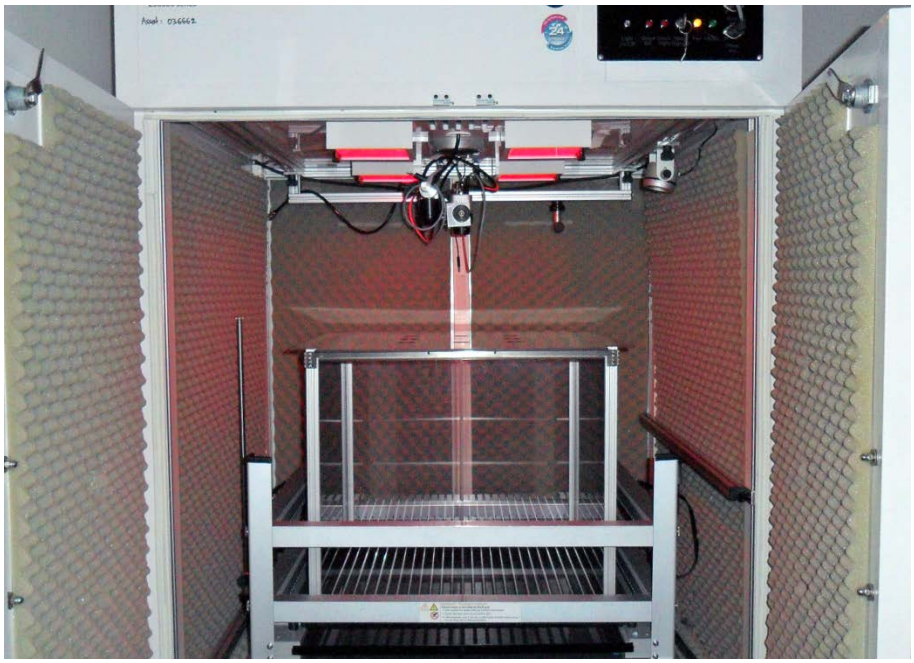

Figure 15. The learned helplessness chamber of the MCS system.

2.3.6.2 Optimization of the LH model

Number of induction sessions

There are two phases in the LH model, including induction (to induce depressive-like symptoms using IS) and testing (the effects of treatment and IS are evaluated using escape shocks) phases. In previous studies, the induction phase was set as either one or two sessions.

In order to induce depressive-like symptoms successfully in male SD rats, different numbers of induction sessions were tested.

In one experiment, a single induction session was used, which contained 60 repeats of inescapable foot shocks (IS, 15 s, 0.8 mA) with a fixed 60 s interval between two shocks. The depressive-like symptoms were assessed in a single test conducted 24 h after the induction phase. In this test, 30 trials of a combination of light (3 s) and ES (3 s, 0.8 mA) was used with a fixed 30 s interval between two trials. The trials were terminated if the animal transferred from one compartment to the other during either light or shock period (**Figure 5A**). The number of trials in which the rats failed to transfer during a 6 s period (= escape failures) was used as an indicator of depressive-like symptoms.

In the other experiment, 2 induction sessions were used on consecutive days. In each induction session, 60 repeats of IS (15 s, 0.8 mA) were combined with a fixed 60 s interval between two shocks. After 24 h, the effects of induction sessions on inducing depressive-like symptoms were measured using 2 testing sessions that were conducted on 2 consecutive days (**Figure 16B**). The same parameters as above were applied in the testing phase of the experiment. The number of escape failures was calculated as indicator of depressive-like symptoms. Three male SD rats were used in each experiment.

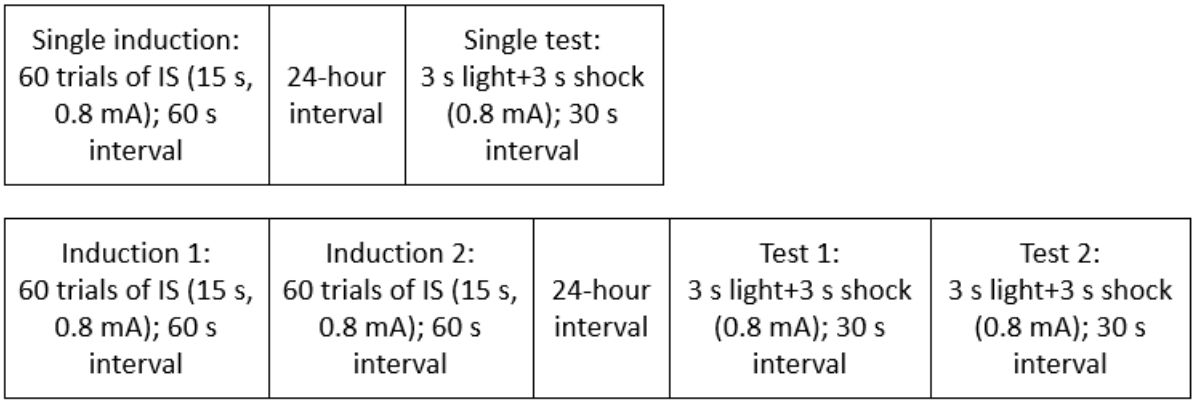

Figure 16. Protocols of comparing different number of induction sessions in the learned helplessness (LH) model.

Number of inescapable foot shocks in the induction phase

In this experiment, the number of IS was increased, in order to enhance the effect of IS to induce long-lasting depressive-like symptoms. Two induction sessions were applied with minor modifications. Male SD rats (n = 3) received 90 IS (15 s, 0.6 mA) with a fixed 60 s interval. The testing phase was carried out 24 h after the 2nd induction session on two consecutive days. In each testing session 30 trials were used, which contained 3 s light, 3 s ES (0.6 mA) and 30 s between two trials (**Figure 17**).

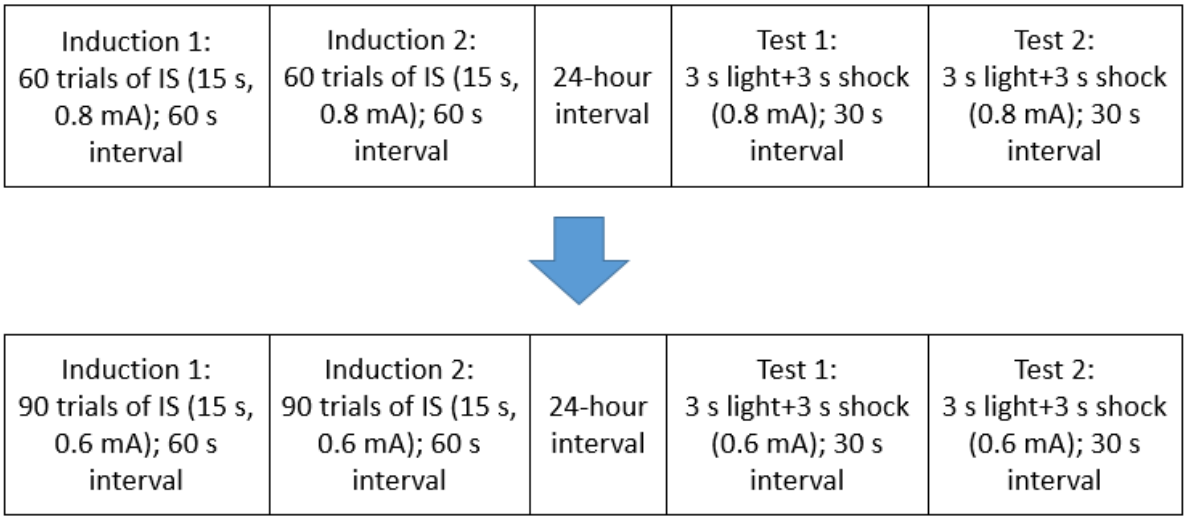

Figure 17. Protocol of comparing different number of inescapable foot shocks in the induction phase of the LH model.

Length of interphase interval

In order to determine the influence of interphase interval on escape performance of rats in the testing phase, the effect of a 48 h interphase interval was tested. Animals (n = 3) were subjected to 2 induction sessions, which contained 90 repeats of IS (15 s, 0.6 mA) with scrambled intervals of 45 – 75 s between two shocks. Three testing sessions were conducted on 3 consecutive days, 48 h after the 2nd induction session. In each test, a combination of 30 trials of 3 s light and 3 s ES was used. Scrambled intertrial intervals of 23 – 37 s was used between

two trials (**Figure 18**). The number of escape failures was calculated as indicator of depressive-like symptoms.

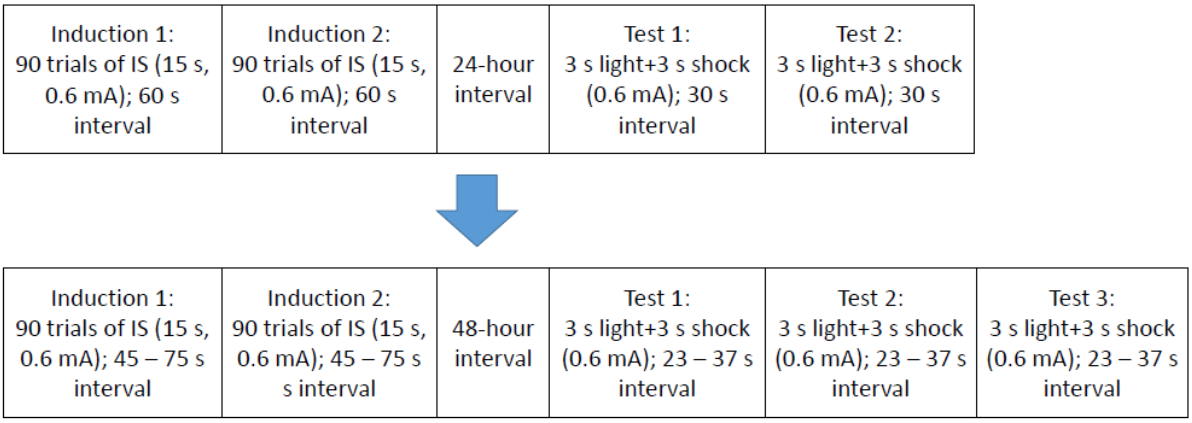

Figure 18. Protocol of comparing the effects of different length interphase intervals in the learned helplessness (LH) model.

2.3.6.3 Application

The modified 6-day LH paradigm included 2 induction sessions and 3 testing sessions with a 48 h interphase interval. The average body weight of male SD rats (n = 5 - 8) was 200 - 310 g at the beginning of the experiments. After being transferred from the Bio-Bubble pavilion to the behavioral room, rats habituated for 60 min to the new environment under 30 Lux illumination every day. Then animals were allowed to explore the LH chamber over a 5 min period before the conduction of the 1st induction session, followed by exposure to 90 IS (15 s, 0.6 mA) with 45 – 75 s intervals between two shocks. This session was carried out without chamber illumination. After 24 h, animals were subjected to another induction session without further habituation in the same chamber with 50 Lux white light illumination. The testing phase was conducted 48 h after the 2nd induction phase. Rats were habituated for 3 min at the beginning of the 1st session, which subsequently consisted of 30 trials of 3 s light and 3 s shock (0.6 mA) with scrambled 23 – 37 s intertrial interval length. The test was repeated two times on consecutive days without further habituation and the same parameters were applied to all animals. Animals were excluded based on their behavioral stress response before and during

the IS paradigm of the LH model in line with previously published report (635). Specifically, in non-stressed rats (NS-group) were excluded if they showed LH symptoms with more than 20 EFs in 30 trials (636). On the other hand, animals of the IS group were excluded if they failed to show LH symptoms and produced less than 10 EFs in 30 trials in the initial LH testing session as previously published (636). Since all measurements were performed in an integrated model, data of excluded rats were removed for all models. The chamber was wiped with F10Sc (1:250 dilution in distilled water) to avoid sensory distraction and cross-contamination between animals. All LH experiments were conducted between 9:00 am and 5:00 pm. Body weight and food consumption were measured daily for the idebenone study.

To illustrate the effects of IS, additional induction sessions with NS and ES were used for two groups of rats. In the NS group, animals were placed in the LH chamber for 20 min without exposure to shocks in the induction phase. In contrast, in the ES group the rats received 90 repeated ES (15 s, 0.6 mA) and shock delivery was terminated if the animal transferred to the other compartment. A further 6 groups of rats that received opioids, imipramine and idebenone treatments based on specific administration regimes (see 2.3.2 for details) after exposure to the IS regimen.

In the LH model, 5 parameters were measured in the testing phase: the number of avoidance events (= number of transfers made during the 3s light), the number of escape failures (= number of trials that rats failed to make any transfers), the number of intertrial interval transfers (= number of transfers made during intertrial intervals), the freezing duration during intertrial intervals and the mean escape latency (= the time taken by the animal to transfer from one compartment to the other from of beginning of trial). These parameters were used as indicators of avoidance learning, depressive-like symptoms, physical activity, anxiety-like symptoms and escape learning respectively.

2.3.7 The light and dark model

2.3.7.1 Apparatus

A two-compartment chamber was used in this LD model. This model consisted of one darkened compartment and a second compartment that was illuminated with 15 Lux white light. The entire chamber was rectangular (450 × 300 × 250 mm) and was partitioned into two equal-sized compartments by a software-controlled automatic door (65 × 70 mm). Exchangeable black walls and a lid surrounded the dark compartment of this chamber, while the other compartment consisted of transparent Plexiglas boards, which allowed white light to penetrate. A square black insert with matte finish was placed underneath the chamber to create an enclosed environment (**Figure 19**).

Figure 19. The light and dark apparatus.

2.3.7.2 Model optimization

A wide range if illumination intensities have been used across different laboratories (637). In order to establish the optimal light intensity for our experimental conditions, a variety of light intensities (0, 5, 15, 30, 50 and 100 Lux) were tested on 6 consecutive days by testing one intensity per day. Male SD rats (n = 3, 7 weeks) were repeatedly used in the experiment. After

habituating to the behavioural room (30 Lux) for 60 min, animals were placed in the centre of the light compartment with the connecting door closed for each session. The animals were allowed to explore the illuminated compartment for 5 min, followed by another 5 min test. During the testing period, the connecting door was opened and rats were able to freely transfer between the two compartments. The percentage of time spent in the dark compartment was used an indicator of anxiety-like symptoms, which was determined as follows:

% time = 100 × (time in seconds spent in dark compartment ÷ 300 s).

The light intensity that did not induce anxiety-like symptoms in naïve rats was selected for subsequent tests. The chamber was wiped with F10Sc (1:250 dilution in distilled water) to avoid sensory distraction and cross-contamination between animals. All tests were conducted between 9:00 and 11:00.

2.3.7.3 Model application

Male SD rats (7 weeks) were habituated for 60 min to the behavioral room (30 Lux) for each session. The rat was placed in the central area of the bright compartment (15 Lux) and was habituated for 5 min with the connecting door closed. Following the habituation, a 5 min test was performed and the door opened automatically. The time spent in the dark compartment was measured over a 5 min testing period. The LD tests were conducted on days 1, 2, and 6, 5 min before the LH procedure (**Figure 20**). The chamber was wiped with F10Sc (1:250 dilution in distilled water) to avoid sensory distraction and cross-contamination between animals.

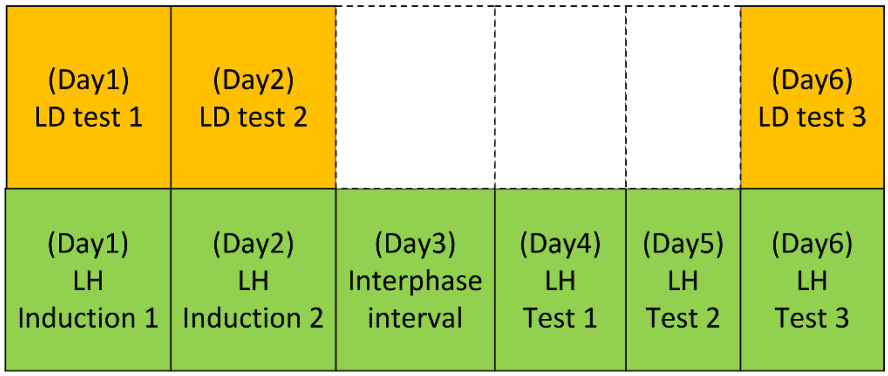

Figure 20. Protocol of the light and dark paradigm (LD).
The LD model was conducted in combination with the LH model. It was performed on days 1, 2 and 6 to measure the anxiety levels of animals.

2.3.8 The open field model

The OF model uses a square open chamber (456 × 456 × 400 mm) with a solid floor plate. It is surrounded by 4 clear Plexiglas walls (**Figure 21A**). The central area of the chamber was defined as a square area representing 50 % of the total chamber size in the center of the arena. In the initial test, after habituating to the behavioral room for 60 min, male SD rats (n = 5 - 8, 7 weeks) were allowed to explore the chamber for 5 min, followed by a 5 min test. Several behaviors were recorded automatically by the software over 5 min testing period: 1) % of time spend in the central area, 2) % of distance travelled in the central area, 3) time spent moving and exploring in the whole chamber, and 4) rearing duration. In addition, the pattern of movement was tracked automatically by the system (**Figure 21B**). The chamber was wiped with F10Sc (1:250 dilution in distilled water) to avoid sensory distraction and cross-contamination between animals.

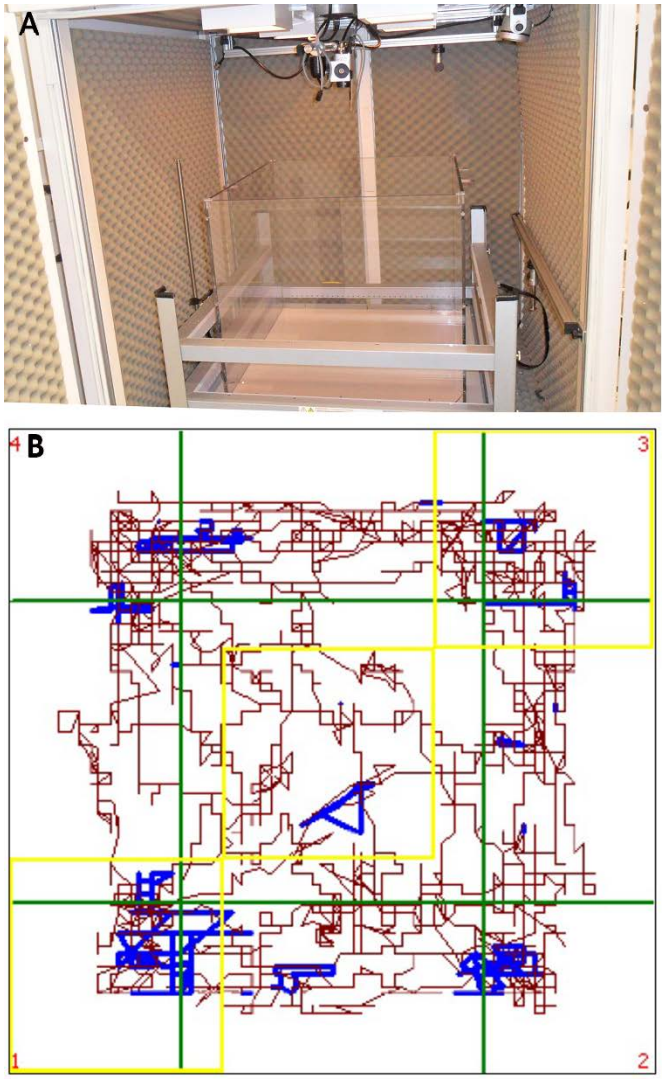

Figure 21. The open field chamber and the movement tracking function of the MCS.
(A) The OF chamber is an open square box with 4 clear walls with a fixed floor plate. (B) The software used in the open field model automatically tracked the moving path of animals. The thin red lines marks their movement and the bold blue line show rearing behavior. The green lines label the corner and central areas of the chamber.

The OF tests were performed 10 min before the LH procedure. In IS, NS (200 µL saline, s.c.) and imipramine (50 mg/kg/day, s.c.) groups, the OF tests were carried out on days 1, 2 and day 6, while for the morphine (5 mg/kg/day, s.c.) group the OF tests were conducted on days 1, 2, 4 to 6 (**Figure 22A**). For the 1001, 1003 (5 mg/kg/day, s.c.) and idebenone (200 mg/kg/day, oral) groups, the OF performance was tested from days 2 to 7 (**Figure 22B**) (see 2.3.2 for treatment administration regimes).

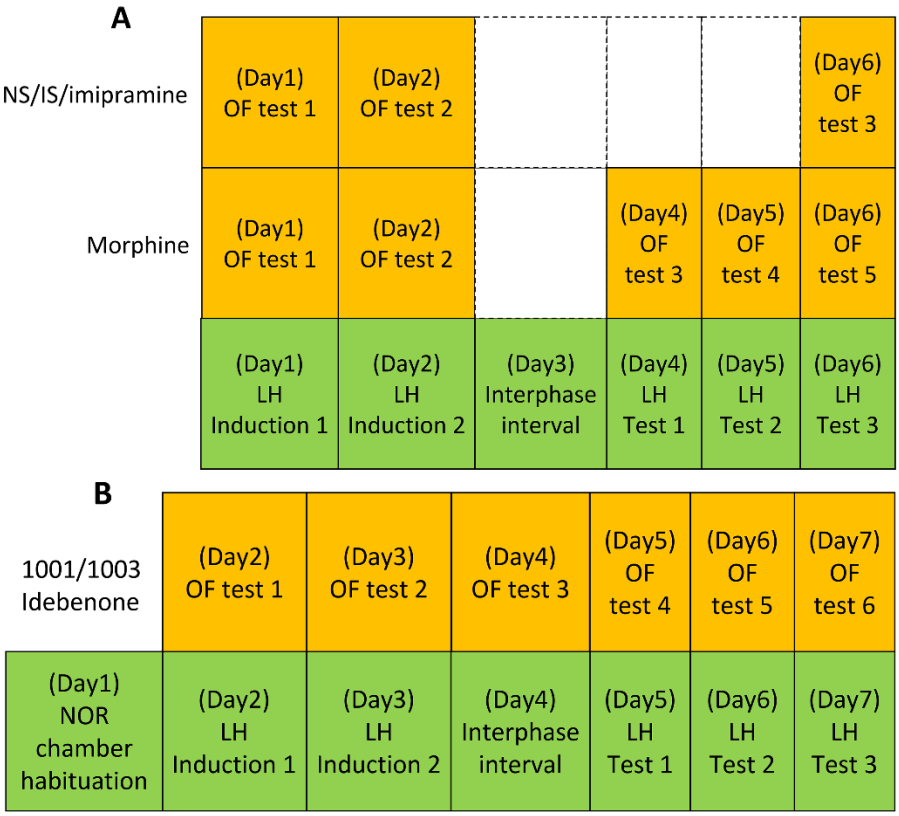

Figure 22. Protocols of the open field paradigm for different groups.

2.3.9 Pain perception

Potential changes to pain thresholds after shock exposure and drug treatment in the IS and IS + morphine groups were measured using the tail-flick and hot plate tests, which are designed to assess heat-induced spinal and supraspinal responses respectively (638) .

2.3.9.1 The tail-flick test

The tail flick (TF) tests were performed parallel to the LH procedure. The intensity of infrared radiation was set to 30 mV / cm^2 to give control times of approximate 4 – 6 sec in male SD rats. Measurement were only performed on a specific part of the tail as sensitivity to radiant heat differs at different parts of the rat tail. Therefore, a distal area of the tail 2 – 4 cm away from of the tip was selected based on previous studies (596,639,640,641). In order to minimise the variance induced by individual tail size, three testing points (i.e., at 2, 3 and 4 cm away

from the tip respectively) were marked within the targeted area.. The rat tail was placed on an infrared beam of the TF equipment as the heat source of the apparatus (**Figure 23**), while the rat's head was covered by a soft black towel. The TF test was only started after sufficient habituation and handling when the animals' tail was completely relaxed on the infrared beam area. After feeling the heat, the animals flicked their tail away from the detector and thus terminated the test. The time to the termination (= tail flick latency) was recorded automatically and was used as an indicator of pain perception. To avoid tissue damage, the maximum cut-off time was set as 15 s. Each rat was measured 3 times for each test at 3 different points on the tails. There was a 1 min interval between each measurement. If a rat failed to cut off the test within 15 s, no further measurement was conducted for the following 5 min.

Figure 23. The instrument used in the tail flick (TF) test.
Tail-flick unit (model 37360) was used in the experiments. Image was copied from the manufacturer's website (*http://www.ugobasile.com/products/catalogue/pain-and-inflammation/item/14-37360-tail-flick-unit.html).*

2.3.9.2 The hot-plate (HP) test

The cold/hot equipment consisted of a metal plate, which was pre-heated to 54 ºC before experiments. A Plexiglas cylinder surrounding the plate prevented the escape of the animal

(**Figure 24**). Male SD rats were placed into the cylinder 15 min after receiving an injection (see 2.3.2 for drug administration regimes). The timer was started manually once the rat was placed on the plate. The test was terminated manually by the operator when the first nociceptive response of licking or shaking a hind paw was observed (= hot plate latency). Each rat was measured 3 times with a 1 min interval between each measurement. A maximum cut-off time of 30 s was set up to avoid tissue damage. If the animal failed to show responses to the heat within 30 s, a 10 min period was allowed before the next measurement was started.

Figure 24. The equipment used in the hot plate (HP) test.
Hot/cold plate unit (Ugo Basile S.R.L. model 37360) was used in the experiments. Image was copied from the distributor's website (*https://www.somatco.com/hotplateforanimal.htm)*.

2.3.10 The novel object recognition model

Novel object recognition is a highly validated test for recognition memory. It has been widely used to test the effects of compounds on both memory enhancement and impairment as well as the influence of genetics or age on memory function (642).

2.3.10.1 Apparatus

The NOR model employs the same chamber as the LH model, where the removable black Plexiglas board is removed to form an open compartment. A black insert is placed underneath the chamber to cover the stainless steel rods (**Figure 25**). There are two phases in this model: the object learning phase (rats learn 2 identical objects) and the memory testing phase (rats are exposed to novel objects). The NOR paradigm was used parallel to the LH procedure.

Figure 25. Arena for the novel object recognition model.
The chamber is an open compartment with black floor insert. The objects are evenly spaced and placed in the middle of the chamber.

2.3.10.2 Model optimization

Selection of objects

In this paradigm, object characteristics such as shape, color and size can *per se* influence the outcome of NOR experiments. The detected object exploration time will be reduced if the tested objects induce stress or fail to attract the attention of the animals (642). Therefore, to ensure the reliability of measurements, these objects should be selected carefully. Preferably, objects should be made from materials that can be easily cleaned and deodorized. In addition, they should be of a size that allows the rats to climb on top, so that they can fully explore the objects. Another important rule for object selection is that the objects should catch the animal's attention and motivate them to explore. For this purpose, 11 different objects were tested (**Figure 26**).

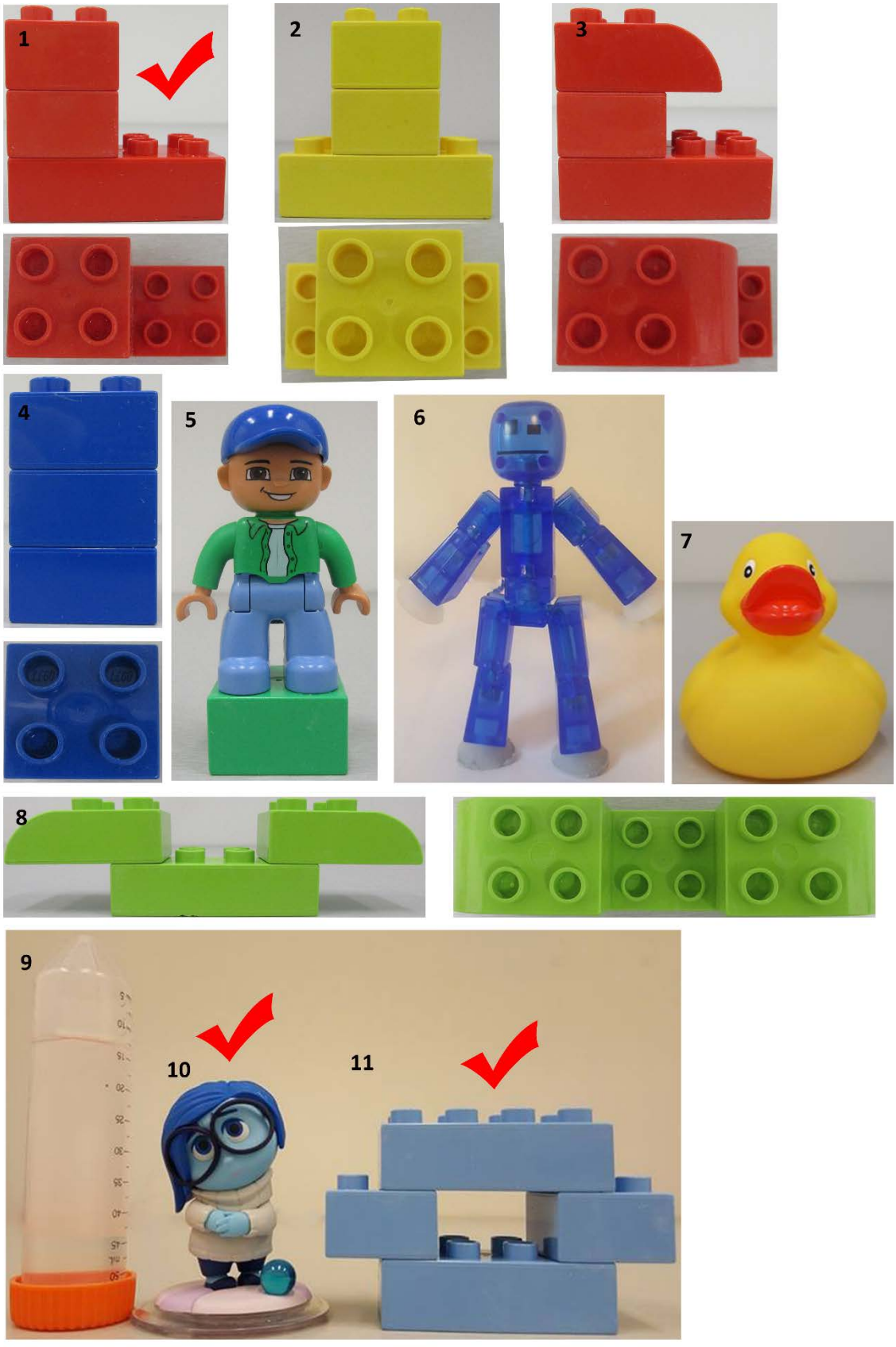

Figure 26. Tested objects for the novel object recognition model.
Eleven plastic objects were chosen and 3 of them (marked with red ticks) were selected for following experiments.

The experiment was conducted between 9:00 - 10:00 am and 4:00 - 5:00 pm on 3 days. In both morning and evening sessions, 2 objects were tested. Selected objects were placed in the middle of the chamber (measured as 22 cm from the top and 14 cm from the side) and their bottoms were attached to the black insert using Blu-tack. Objects were evenly spaced (around 17 cm between two objects) every time (**Figure 27**).

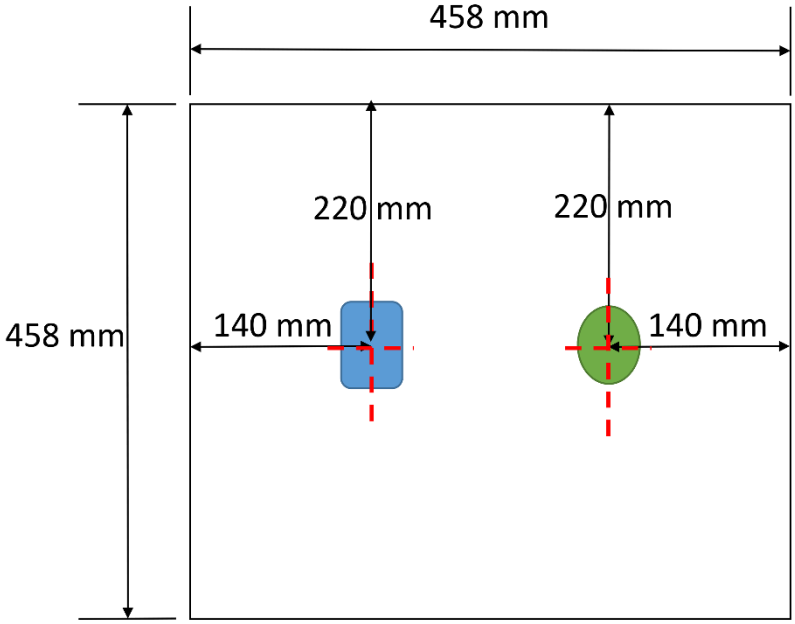

Figure 27. Location of objects in the NOR chamber.

Animals (n = 4) were allowed to explore the objects for 10 min in each session. Time spent sniffing, touching with nose tip or forepaws whilst sniffing and climbing was recorded manually using a stopwatch and considered as exploration time. However, time spent turning around, leaning against and standing on the objects was excluded (643). At the end of these trial experiments, three objects (marked by red ticks in **Figure 26**) where the rats spent the most time exploring were selected for the following experiments. F10Sc was used to clean the objects between each rat to avoid sensory distraction and cross contamination, followed by 70 % ethanol to remove the odor.

Persistence of the natural memory of rats

Because IS can induce memory loss in rodents (644), the natural memory of rats (n = 3) was tested without exposing them to the foot shocks. This experiment contained two learning sessions and four testing sessions with a 24 h interval between two sessions. In the learning session, 2 identical objects were used. Rats were allowed to explore the objects for 20 min in each learning session on days 1 and 2. Over days 3 to 6, the memory of the animals was assessed 24, 48, 72 and 96 h after the 2nd learning session respectively. In each testing session, one of the familiar objects was replaced with a novel object. Rats were exposed to both objects for 5 min. The experiment was recorded to DVD disk, which was subsequently analysed using VideoMot 2 software. The actual time spent exploring both novel and familiar objects was calculated and the exploration time for the novel object was used an indicator of memory function.

Effect of stress exposure on learning and memory

Cognitive dysfunction has been frequently reported in patients with depression and has been replicated in depressed rodents in the LH model (87,644). However, stress exposure can either enhance or impair memory dependent on the interval between stress exposure and object learning (645). Therefore, to produce symptoms of memory loss in our LH model, 2 experimental designs were tested in which the learning sessions were conducted at different time points after exposing the rats to stress. In the first experiment (**Figure 28A**), rats (n = 3) habituated to the NOR chamber for 30 min before on day 1 before they were subjected to 90 IS on day 2. Ten min after the shock exposure, a 20 min object learning session was carried out using 2 identical objects. On day 3, rats were given another 90 IS, followed 10 min later by

the 2nd 20 min learning session. The memory of animals was assessed on day 4 in a 5 min testing session using a novel object.

In the second experiment (**Figure 28B**), rats (n = 3) were habituated to the NOR chamber on day 1, before exposing them to 90 IS on days 2 and 3. Two object learning sessions were conducted 24 h after the 2nd IS session using the identical objects used in experiment 1 on day 4 with a 7 h interval between two sessions. The memory of rats was initially measured 30 min after the 2nd object learning session over a 5 min period. Another two 5 min memory testing sessions were conducted on days 5 and 6. Different novel objects were used in different testing sessions. Both experiments were recorded automatically to DVD and subsequently analysed using VideoMot 2 software.

Days	1	2	3	4	5	6
A	Habituation	IS1+L1	IS2+L2	T1		
B	Habituation	IS 1	IS 2	L1 & L2 & T1	T2	T3

Figure 28. Two post-stress learning paradigms of the novel object recognition test.

2.3.10.3 Model application

The modified NOR model was performed over 7 days in parallel to the LH paradigm (**Figure 29**). The NOR chamber was illuminated with 15 Lux white light during the procedure. On day 1, rats were habituated to the NOR chamber for 30 min before they were transferred back to Bio-bubble pavilion. Approximately 24 h after the habituation, animals received the 1st LH induction session in an unilluminated chamber, which contained either 90 trials of IS (15 s, 0.6 mA) with an average of 60 s scrambled intertrial intervals or 20 min non-shock exploration time. The foot-shock exposure was repeated on day 3 and on this day, the chamber was illuminated with 50 Lux white light. On day 4, rats were subjected to 2 x 20 min learning sessions using two identical objects (object #1 was used in the learning phase see **Figure 26**),

with a 7 h interval between two sessions. On day 5, the 1st NOR test was performed over a 5 min period by replacing one of the familiar objects with object #10 (see **Figure 26**), 15 min after the injection of 1001 and 1003. On the evening of day 5, the post-learning idebenone group received the first dose of 200 mg/kg/day idebenone through their food (see 2.3.2 for drug administration regimes). On day 7, long-term memory was assessed 15 min after the drug injection in a 5 min test using object #11 (see **Figure 26**). The experiments were recorded and then analysed using VideoMot 2, to determine the exploration time for the different objects.

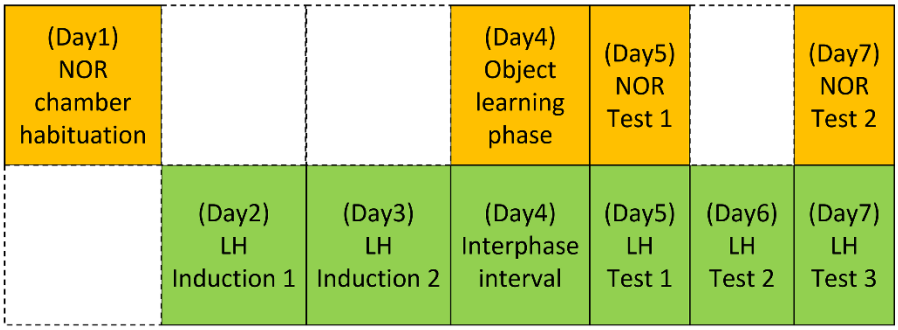

Figure 29. Protocol of the novel object recognition model .

2.3.10.4 Discrimination index

To assess differences in exploring time for the familiar and the novel objects in a memory testing session, the commonly used DI was determined using the following formula:

$DI = (T_N - T_F) / (T_N + T_F)$

(T_N: exploration time on novel object, T_F: exploration time on familiar object).

A value below zero indicates that the animals explored the familiar object more than the novel object. A value above zero indicates that the animals explored the novel object more than the familiar object. The use of the DI allows to identify differential exploration behaviors for both objects (642).

2.3.11 The fear extinction model

The FE model was developed to investigate the neural circuits of extinction of FC in both animals and humans (646). In the current study, this model consisted of 4 phases: habituation, FC training, FE training and a post-extinction (PE) test. Different chambers were used at different phases of this model, in order to minimize the fear that associated with specific experimental environments.

2.3.11.1 Model optimization

In this preliminary experiment, the most commonly used parameters described in previous studies were tested. Similarly, 2 different chambers were used for the different phases of the FE model. For the habituation and FC phases, the chamber used in the LH model without the black board in the middle was employed (**Figure 30A**). On day 1, rats (n = 4) were placed inside this chamber and allowed to explore the chamber for 20 min. After 24 h, animals were placed back to the same box and were given 20 min for habituation before being subjected to six stimuli pairs of tone (20 s, 68 db, 10 kHz) and shock (0.5 s, 0.8 mA), which co-terminated with the tone. The trials were separated by an intertrial interval of 100 ± 20 s. After exposure to the tone and foot shock, rats were kept in the chamber for a further 2 min before returned to their housing cages. No illumination was provided during the habituation and FC phases. Chamber 1 was wiped with F10Sc after each rat. On days 3 and 4, rats received FE training in chamber 2, which was illuminated with 15 Lux white light (**Figure 30B**). To create a different experimental environment, chamber characteristics were changed based on odour, visual and tactile cues. The chamber was changed to a shorter rectangular size (450 × 300 × 250 mm) with black/white stripes on the front wall and 15 Lux white light illumination. On the floor of the chamber, a layer of paper towels and bedding materials was provided and sprayed with 70% ethanol to offer a distinct smell. Chamber 2 was wiped with F10Sc followed by 70 % ethanol

after each rat. In the first FE session, rats were placed into the new chamber, where they were exposed to 30 trials of the same tone signal (68 db, 10 kHz, 20 s) with 100 ± 20 s intervals. After 24 h, animals were exposed to a FE session of 15 trials using identical settings. On the last day, a PE testing phase was conducted by exposing the animals to 6 trials of the same tone signals in chamber 2 without illumination. The freezing duration during the tone signal was recorded and used as an indicator of fear.

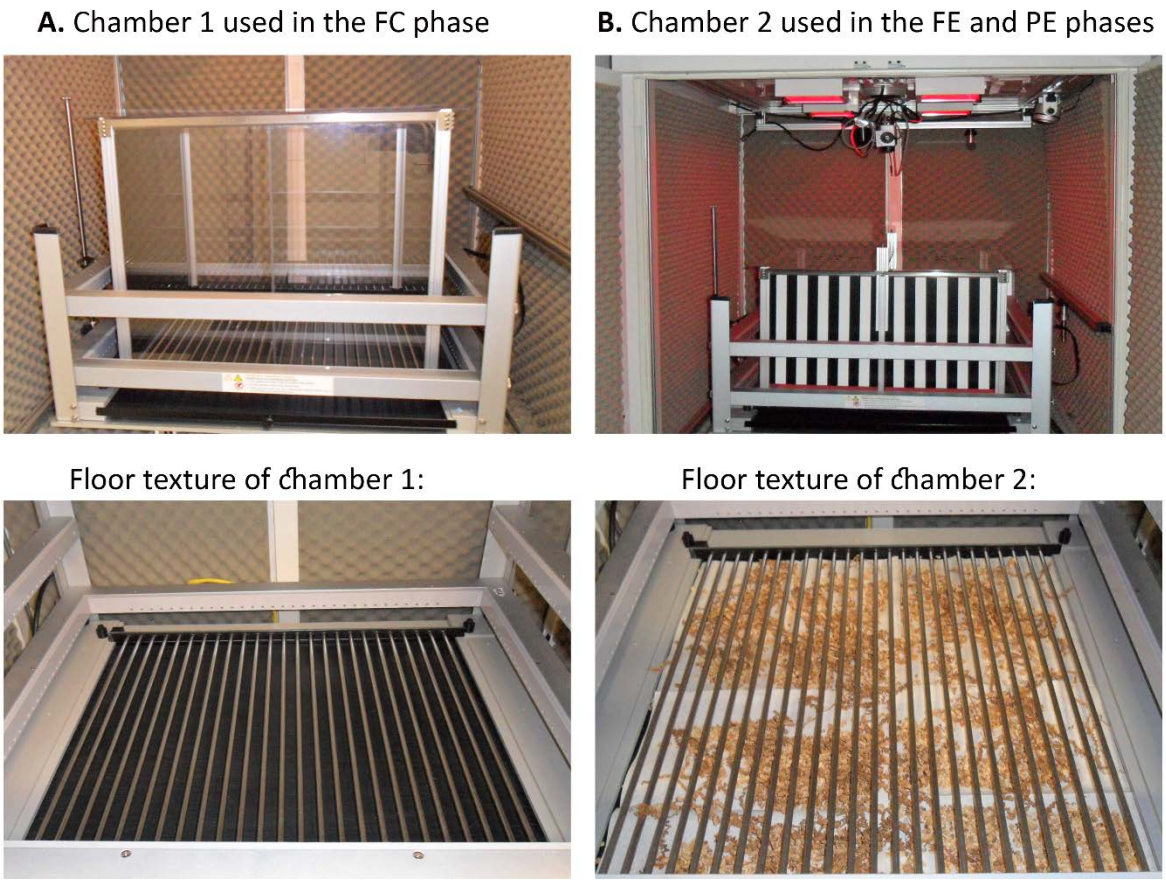

Figure 30. Two chambers that used in the optimization experiment of the fear extinction (FE) model.

2.3.11.2 Model application

In this experiment, chamber 1 was used (**Figure 31A**). However, in order to increase the discrimination between 2 chambers and to reduce the fear of rats towards the grids, a grey insert

with a smooth surface was placed underneath the chamber to cover the steel grids (**Figure 31B**).

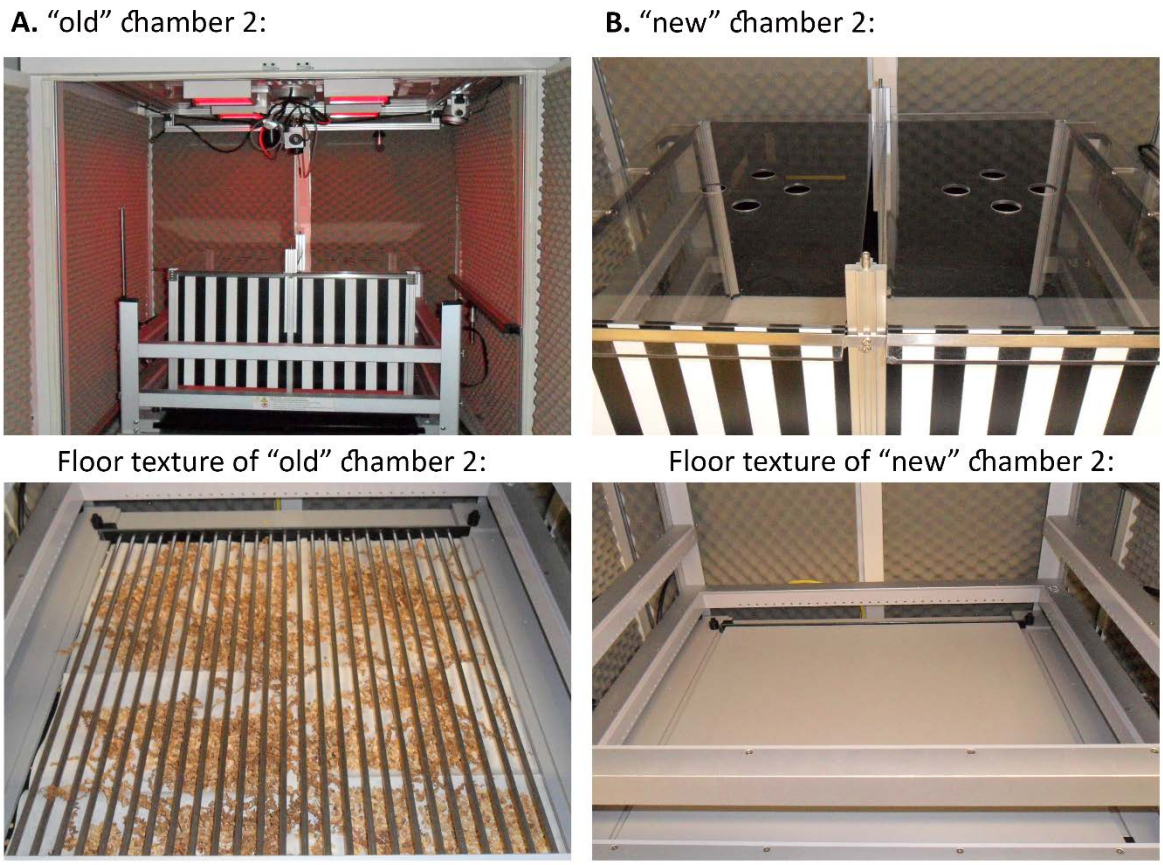

Figure 31. The changes made to chamber 2 of the fear extinction (FE) model.

Rats were given a daily dose of idebenone (200 mg/kg/day, see 2.4.1.2 for details of idebenone preparation) between 5:30 and 6:00 pm for 7 days prior to the conduction of the FE procedure. The drug was continuously administrated until the end of day 3. To minimize the stress associated with the transfer from the Bio-Bubble pavilion to the behavioral room, the animals were transferred once a day for 3 days prior to the experiment. Each day animals were habituated to the behavioral room for 30 min under 30 Lux illumination before being returned to the Bio-Bubble pavilion.

Minor changes were made to the protocol used in the preliminary study, in order to enhance the effect of FC training to generate fear towards the tone. Briefly, on day 1 rats were habituated

to chamber 1 for 30 min before returned to their home cages. On day 2, after another 2 min habituation period, they were exposed to six pairs of tone (30 s, 75 db, 4 kHz) and shock (1 s, 0.8 mA, co-terminated with the tone) with a scrambled intertrial interval of 100 ± 50 s between pairs. Chamber 1 was cleaned with F10Sc between rats. For FE training, rats were presented with 8 trials of the same tone signals with the same duration of intertrial interval in chamber 2, followed by another 2 min delay before placing them back to their home cages on day 3. Chamber 2 was wiped with 70 % ethanol between animals. The PE test was performed approximately 24 h after the FE training (**Figure 32**). Red light illumination (15 Lux) was used throughout the experiment.

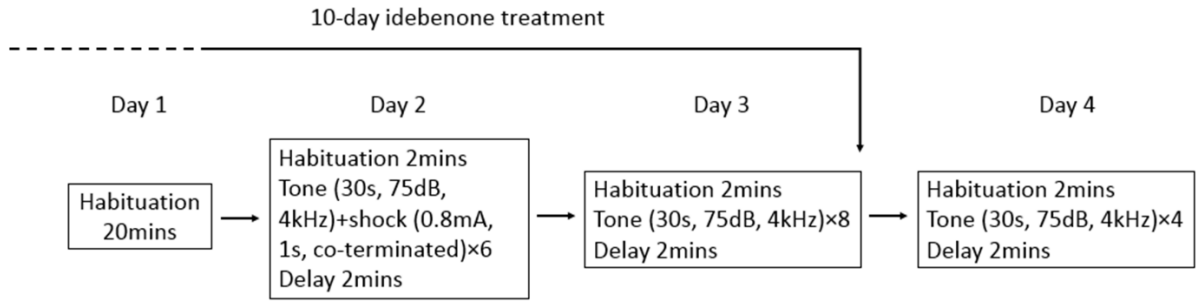

Figure 32. Protocol of the fear extinction (FE) model.

2.3.11.3 Data collection and analysis

Freezing behaviour is defined as the lack of movement except movement required for respiration. In the experiment, 'freezing duration' was recorded when the freezing behaviour of rats was greater than 3 s and encompassed the total length of time the rat kept still (647). Freezing response is represented as percentage of freezing duration in response to a 30 s tone. The freezing response is automatically recorded by the FC software of the MCS in seconds, while the freezing (% time) of each rat is calculated manually by adding up all recorded freezing times during a tone signal and then divided by 30.

2.3.12 Transcardiac perfusion fixation

2.3.12.1 Equipment and preparation of anesthetics

2.3.12.1.1 Isoflurane stinger

A mobile isoflurane stinger contains isoflurane vaporizer, oxygen supply, gas supply regulator and flowmeter (0 – 1000 mL/min). The system was checked before each use to ensure adequate amounts of supply gas and isoflurane were available for the following procedure. Then the connection between induction chamber and the stinger was checked to ensure adequate flow of anesthetic gas into the chamber. After this system check, both oxygen supply and isoflurane vaporizer were turned on.

2.3.12.1.2 Perfusion bench and peristaltic pump

The perfusion fixation procedure was performed in a fume cupboard. A plastic box with steel net on the top was used to support the rat body and to collect blood. Bottles of ice cold sterilized PBS and 4 % PFA in PBS were placed on the side of the peristaltic pump. The tubes of the perfusion pump were flushed with PBS to remove residual bubbles before use. The flow rate for PBS and PFA was set as 20 mL/min. In addition, 50 mL centrifuge tubes with ice cold 4 % PFA solution was placed inside the fume cupboard for brain tissue collection.

2.3.12.1.3 Pentobarbital sodium

Stock solution of pentobarbital sodium (325 mg/mL) was diluted with sterilized saline to 60 mg/mL and used at a dose of 200 mg/kg.

2.3.12.2 Perfusion fixation

Rats were placed in the induction chamber and anesthetized using 5 % isoflurane delivered at a rate of 800 mL/min. Once the animal was anesthetized, pentobarbital sodium (200 mg/kg at

60 mg/mL) was injected (i.p., 29G ½" needle). After the absence of pedal reflex was confirmed, a wide incision was made with a scalpel through the abdomen. With sharp scissors, the connective tissue at the bottom of diaphragm was cut to allow access to the rib cage. Then the thoracic cavity was opened up with larger scissors along the sidelines of the rib cage and clamped open to expose the heart and to provide drainage for blood and fluids. While holding the beating heart steady with forceps, the perfusion needle was inserted vertically directly into protrusion of the left ventricle and secured in this position by clamping near the point of entry. As soon as an incision was made in the atrium with sharp scissors to allow circulation, PBS was pumped through the rat's body. Then 4 % PFA was delivered to fix the brain tissue. Both PBS and PFA were delivered at a rate of 20 mL/min. The perfusion fixation was terminated when pale eyes and a stiff body were observed. At the end of the perfusion fixation, brain tissue was collected into 50 mL centrifuge tubes. The brain samples were kept at 4 ºC overnight for immersion-fixation, before proceeding to dehydration and embedding.

2.3.13 Blood collection

2.3.13.1 Preparation of EDTA-coated tubes

Blood was extracted from the rat's heart immediately prior to perfusion fixation and collected using 0.5 M EDTA (pH 8.0) -coated 1.5 mL Eppendorf safe-lock tubes. EDTA powder was dissolved in MilliQ water to prepare a 0.5 M stock and adjusted to pH 8.0 before autoclaving. The final solution was stored at room temperature. This 0.5 M EDTA solution was used as anticoagulant. EDTA-coated 1.5 mL Eppendorf tubes were prepared fresh by adding 1.5 mL EDTA into the tubes. After 1 min, EDTA was transferred into other tubes in sequence. In the last, 0.5 M EDTA was loaded into the coated tubes at an EDTA/blood ratio of 1:100. All tubes and pipette tips were pre-cooled to 4ºC.

2.3.13.2 Plasma collection

In present study, plasma was collected to quantify the levels of stress hormones such as cortisol as indicator of HPA axis function. Before inserting the perfusion needle into the heart, blood was extracted and transferred into the EDTA-coated 1.5 mL Eppendorf tubes. Then the blood sample was centrifuged immediately at 10,000 × g for 90 s at room temperature. Supernatants were stored at -80 ºC for further analysis.

2.3.14 Statistical analysis

The data of all behavioural tests were automatically recorded by the MCS software. Recorded videos from the novel object recognition model were analysed using VideoMot 2. The *in vitro* and *in vivo* data were expressed as mean ± standard deviation (SD) and mean ± standard error (SEM) respectively, as indicated in the figure legends. Determination of statistical significance was performed using Student *t* test, (repeated) one-way or (repeated) two-way Analysis Of Variance (ANOVA) followed by Tukey's or Dunnett's multiple comparison tests where appropriate using GraphPad Prism. Images obtained from histological analysis were analysed using Fiji ImageJ. In all assays, $p<0.05$ was considered as statistically significant and the results were represented as mean ± SEM, in presence of the value of degrees of freedom DFn (= a – 1, where a is the number of groups) and DFd (= N - a, where N is the total number of subjects in all groups). All figures were assembled using Photoshop CS6.

2.4 *Ex vivo* study

2.4.1 Western blot

2.4.1.1 Materials

Rats that were used in the morphine and imipramine study were decapitated straight after isoflurane anesthesia (see 2.3.10.1.1 for isoflurane vaporizer setup). The brain was removed

immediately into a cryotube and snap-frozen in liquid nitrogen, before stored at -80 ºC for western blot (WB) analysis. To determine the impact of the snap-freezing procedure on protein stability, fresh tissue was also tested as in parallel. Snap-frozen rat liver microsomes were donated by another group and used as positive control for the analysis.

2.4.1.2 Protein extraction and sample preparation

Both hemispheres of the brain were cut into 3 sections in coronal direction, including anterior (½) cerebrum, posterior (the other ½) cerebrum and cerebellum. Ice cold protein lysis buffer (50 mM Tris-HCl, pH 7.4, 1 % NP-40, 0.5 % Na-deoxycholate, 0.1 % SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 µM DTT, 100 µM PMSF and 10 µM Na_3VO_4) was added to the weighed tissue at a ratio of 8 µL/mg tissue. The tissues were homogenized manually on ice using a pestle method, before being transferred into 1.5 mL Eppendorf tube for vortexing over a 15 min period at 4 ºC. Then the lysate was homogenized further using an ultrasonic homogenizer (3 x 10 s, with 1-2 min intervals). The resulting lysate was centrifuged at 22,000 × g for 20 min at 4 ºC. The protein content in the supernatant was determined as described in 2.2.2.2. The supernatant was diluted with protein lysis buffer and 4x Laemmli buffer (40 % glycerol, 240 mM pH 6.8 Tris-HCl, 8 % SDS, 0.04 % bromophenol blue, 5 % beta-mercaptoethanol) to a concentration of 5 µg/µL and stored at -20 ºC until use.

2.4.1.3 Gel electrophoresis

Eight percentage of gels were cast (375 µM Tris pH 8.8, 7.8 % acrylamide, 0.1 % SDS, freshly added 0.1 % APS and 0.1 % TEMED) in a commercial mini-gel apparatus. 1 mL of isopropanol was layered on top of the separating gel to smooth the border and avoid exposing the top of gel to oxygen. After 20 min, isopropanol was removed and the top of the gel was cleaned with milliQ water. The stacking gel (126 µM Tris pH 6.8, 3.9 % acrylamide, 0.1 % SDS and freshly

added 0.15 % APS and 0.14 % TEMED) was poured on the top of the separating gel after the inside of the glass plates was dried with filter paper. A comb was positioned into the stacking gel. After 10 min, the comb was slowly removed and the wells were rinsed thoroughly with MilliQ water to remove non-polymerized acrylamide. Running buffer (25 mM Tris base, 192 mM glycine, 10 % SDS) was partially loaded into the wells for the ease of loading samples. 10 µL of 5 µg/µL protein samples were denatured for 5 min at 95 °C and loaded into each well. The gel cassette was then placed into the electrophoresis chamber, which was filled with running buffer (7 mM SDS, 50 mM Tris and 38.4 mM glycine, pH 8.3). The proteins were separated at a constant 25 mA per gel. Protein transfer from separating gel onto a nitrocellulose membrane was carried out by assembling a sandwich of gel, membrane and Whatmann filter paper cut to 5.3 × 8.1 cm. The sandwich was placed inside a holder and submerged in the apparatus with transfer buffer (25 mM Tris base, 192 mM glycine, 10 % SDS, 20 % methanol) with the gel facing the anode. The transfer was performed at a constant voltage (100 V) for one hour at 4 °C. Subsequently, the membrane was briefly washed in MilliQ water and then blocked using 5 % skimmed milk powder in Tris-buffered saline with 0.1 % Tween-20 (TBS-T) for 1 h, before incubation with anti-MAOA antibody solution (ab126751, 1:1000 in blocking buffer) overnight at 4 °C. The next day, the membrane was washed 3x 10 min with TBS-T and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (ab97051, 1:5000) in blocking buffer for 1 hour at RT. The membrane was washed 4x 10 min with TBS-T. Antibody binding was visualized using an Amersham Imager with enhanced chemiluminescence western blotting reagent according to the manufacturer's instructions.

2.4.2 Histology

2.4.2.1 Tissue fixation and dehydration

All brain tissues were dehydrated in 70 % ice cold ethanol for 7 days at 4 ºC, after being immersed in 4 % PFA (in PBS, pH 7.4) overnight.

2.4.2.2 Tissue processing

Table 5. Processing steps of rat brain tissue for histology.

Solution	Duration (Min)	Temperature (˚C)
Ethanol 70 %	60	37
Ethanol 95 %	60	37
Ethanol absolute	60	37
Ethanol absolute	90	37
Ethanol absolute	90	37
Ethanol absolute	120	37
Xylene	60	37
Xylene	60	37
Paraplast wax	60	60
Paraplast wax	60	60
Paraplast wax	60	60

The rat cerebellum was removed before briefly dissecting the remaining tissue into four portions at 10 mm, 12 mm, 14 mm and 16 mm respectively away from the top of the olfactory bulb. Among these portions, the ones containing the hippocampus were kept for further processing. In such way, the hippocampus was divided into 2 parts, which separately contained the anterior and the posterior areas of the hippocampus. The dissection points were selected based on the online Rat Atlas tool (http://labs.gaidi.ca/rat-brain-atlas/). Tissue was processed using a Leica ASP200 auto-processor. To ensure the success of paraffin wax infiltration, water was completely removed following a series of dehydration steps using increasing concentration of ethanol. The tissue was infiltrated with paraffin wax after being cleared in xylene (**Table 5**).

2.4.2.3 Embedding and sectioning

Two parts of the hippocampal rat brain were embedded into a single paraffin wax block using a metal mould. A Leica 2250 microtome was initially adjusted to 10 µM for coarse sections followed by 5 µM sections when the anterior hippocampus was reached (**Figure 33**). Every 100 sections was considered as a group and labelled in alphabetical order, thus approximately 400 sections were produced. Due to limited availability of antibody, only the 50th section of the second group was collected for analysis. Therefore, 5 - 8 slides were stained for each group (one slide per rat).

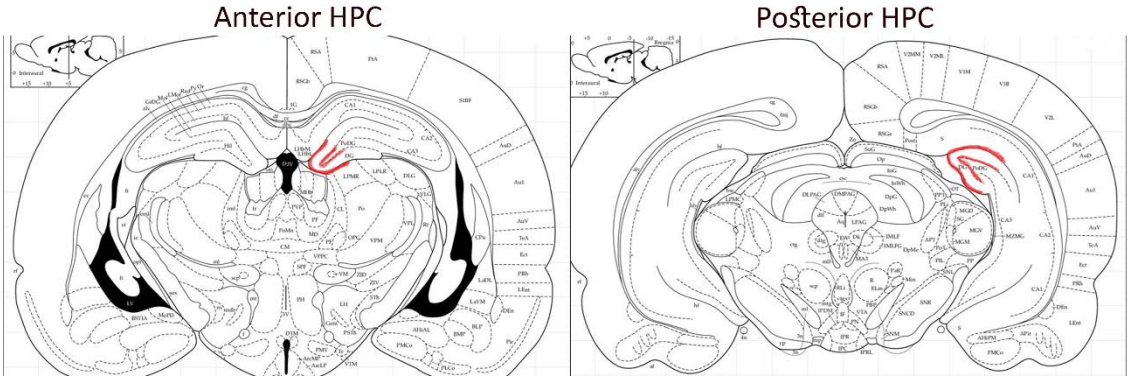

Figure 33. Brain area of interest.
Both anterior and posterior hippocampal sections were collected and the dentate gyrus region was focused on for the histological analysis (as marked by red lines).

2.4.2.4 Immunohistochemistry

Paraffin sections were placed in a heater at 60 ˚C for 20 min to melt the wax and help specimen attachment to the Dako IHC slides. Then sections were dewaxed and rehydrated in fresh xylene twice for 10 min each, 100 % ethanol, 95 % ethanol, 70 % ethanol, MilliQ water and RT PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO4, pH 7.4) for 5 min each. Then antigen retrieval was achieved by heat (pressure cooker method in 10 mM citrate buffer pH 6.0 for 20 min), followed by 3 washes with PBS for 10 min each.

2.4.2.4.1 Fluorescent IHC staining

After PBS washing, sections were blocked with 10 % horse serum and 0.2 % Triton X-100 in PBS for 1 h at RT, before incubation with either primary antibodies against MAO-A (ab126751, 1:200) in antibody diluent (1 % horse serum, 0.2 % Triton X-100 in PBS) or blocking solution (200 µL per slide) overnight at 4 ˚C. After the sections were rinsed three times with PBS-T (0.1 % Tween-20 in PBS) for 10 min each, they were allowed to react with goat anti-rabbit Alexa Fluor® 594 (ab150084, 1:2000) for 2 h at RT, followed by another 3 washes with PBS-T. The sections were subsequently incubated with DAPI solution (1:5000 in PBS) before being mounted with Dako florescent mounting medium and left to dry overnight. Nikon NIS-Elements D413 (64-bit) suite was used to acquire fluorescence images. Digital images were mounted into panels and labelled with Adobe Photoshop Software CS6.

2.4.2.4.2 DAB immunohistochemistry

After PBS washing, sections were incubated with 1 % hydrogen peroxide for 5 min at RT to block endogenous peroxidase activity, followed by three washes with PBS for 5 min each. To block non-specific background staining, the sections were blocked (4 % milk, 10 % horse serum, 5 % BSA, 0.2 % Triton X-100 in PBS) for 1 h at RT before incubating with primary antibody against nitrotyrosine (A21285, 1:300) in antibody diluent (2 % milk, 1 % horse serum, 0.2 % Triton X-100 in PBS) overnight at 4 ˚C. Then sections were reacted with biotinylated anti-rabbit IgG (PK-4001, 1:200) for 1 hr at RT before incubated with ABC solution (PK-6100) for 30 min. The histological signal was developed by incubating the sections with DAB solution (3:100) for 5 min, followed by counterstaining with hematoxylin (protocol see 2.4.2.4.3). The sections were dehydrated (fresh 90 % ethanol, absolute ethanol twice and xylene 1 and 2 for 5 min each) and mounted using an auto-coverslipper. Finally, the mounted sections

were examined using light microscopy (Leica DM 2500). Digital images were assembled into panels and labelled with Adobe Photoshop Software CS6.

2.4.2.4.3 Hematoxylin staining

The sections were incubated with Dako hematoxylin solution for 5 min and washed in running water until the tissue turned pink (color change was monitored using light microscopy). Then the sections were exposed to 0.2 % ammonia water for 30 s before washed in running water until a blue color developed.

2.4.3 Imaging analysis

DAB immunohistochemistry and fluorescent IHC images were acquired using light and fluorescence microscopy respectively at 20 x magnification. Images were analysed using Fiji ImageJ. Briefly, the color of all images was changed to greyscale as the initial step. Then the region of interest (ROI) was manually selected on the acquired images based on the anatomic structure of the HPC, by outlining the granular cell layer of the hippocampal DG. Threshold adjustment is an essential step in imageJ analysis to segment grayscale images into features of interest and background. To obtain uniform threshold values for all images, initially the staining intensity of the negative control was examined using imageJ. For this, both lower and upper thresholds were adjusted to identify the threshold values that would not pick up any staining signals from the image of negative control. Subsequently, individual lower and upper threshold values of the samples stained with the primary antibody were adjusted based on the identified threshold values of the negative control. Using this approach, the lower and upper threshold values were set to 48 and 116 respectively for all analyzed images. This procedure ensured that only intense staining in dark grey to black color was detected, while pale signals of comparable intensity to the negative control were excluded (**Figure 34**). Any images that

would have required different thresholds were excluded from the analysis. In the last step, the ratio of positive staining in relation to the ROI was determined and the results were presented as % area. For immunohistochemical quantification, blinding to the treatment groups was not performed because ROI selection was fully based on anatomical structure, which was clearly visible in the images with very little scope for investigatot bias. In addition, oxidative damage was scored using the image analysis method described above with little potential for investigator bias.The data was further analysed using GraphPad Prism (Version 6) and the average values calculated from 5-8 slides per group was used to represent the data.

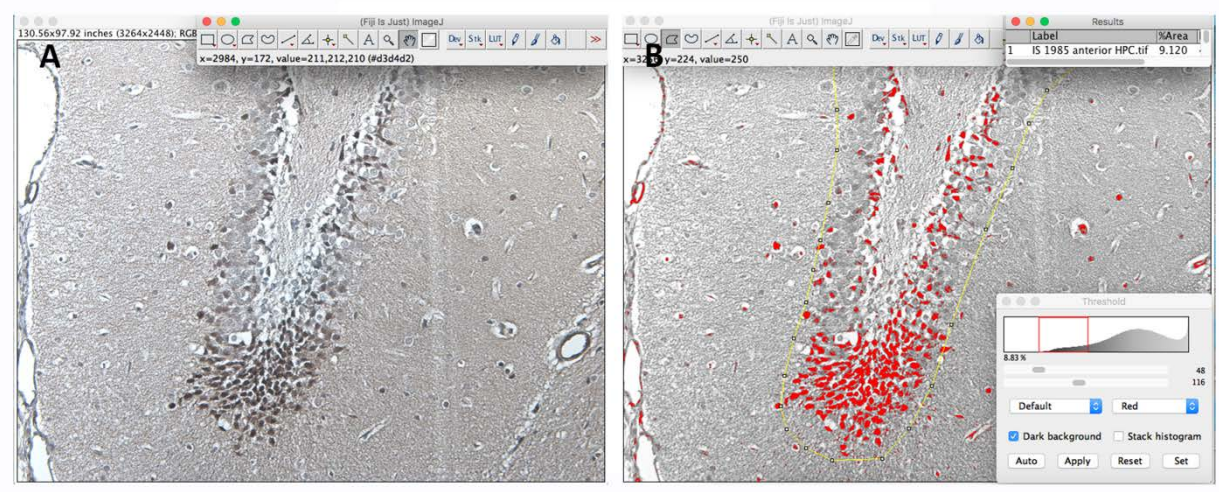

Figure 34. Quantification of IHC images using Fiji ImageJ.
Three basic stages to analyze histological staining images were used that included changing image color, setting contrast and threshold and selecting an area of interest.

Chapter 3 Optimisation of Behavioural Animal Models

Based on the reported antidepressant-like effects of both clinical used and experimental opioids (288,332), we aimed to test the effects of novel UTAS-synthesized bifunctional opioids on depression and depression-associated comorbidities such as stress-induced analgesia, anxiety and cognitive dysfunction. To ensure the validity of the results, all behavioural models needed to be optimised before drug-induced effects on animal behaviours could be determined.

3.1 Optimising the light and dark model

Anxiety-like symptoms were assessed in the LD model (648). This model uses a two-compartment chamber, of which one compartment is illuminated with white light while the other is darkened. To optimise the experimental settings, different light intensities were tested in this model using male Sprague Dawley (SD) rats (n=3) over a period of 5 min. With increasing light intensities between 0 and 100 Lux, the animal spent an increasing amount of time in the darkened compartment, indicative of increased levels of anxiety-like symptoms. From 30 Lux onwards, animals spent significantly more time in the darkened chamber compared to basal levels at 0 Lux (repeated 1-way ANOVA, 30 Lux: (70.79 ± 6.156) s, p=0.00028; 50 Lux: (97.45 ± 4.827) s, p<0.0001 and 100 Lux: (99.30 ± 2.476) s p<0.0001) (**Figure 35**).

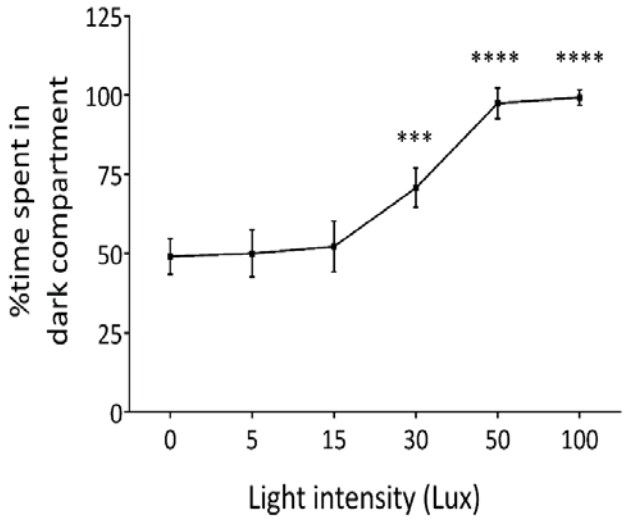

Figure 35. Optimisation of the light and dark (LD) model.
The impact of light intensity on anxiety-like behaviour of rats was investigated in the LD model using a two-compartment chamber. The experiment was conducted on 6 consecutive days with the same animals (n=3) by testing one light intensity per day. The data represents as percentage of time spent in the dark compartment over a 5-minute period. $^{***}p$<0.001 and $^{****}p$<0.0001 versus basal level at 0 Lux using repeated one-way analysis of variance (ANOVA) followed by Dunnett comparison tests. Error bar=SEM.

3.2 Optimising the learned helplessness model

Depressive-like symptoms of rats were assessed using the LH model, which exposes animals to IS. Three crucial parameters, the number of induction sessions, the number of shocks and the length of the interphase interval were optimised using an automated MCS. Learned helplessness occurs when an animal is repeatedly subjected to an aversive stimulus that it cannot escape. As a consequence, the animal will stop trying to avoid the stimulus even when they are allowed to escape. This unwillingness to escape is indicative of depressive-like symptoms and is quantified as the number of escape failures (EF). In the first experimental design, rats were only subjected to one induction session, containing 60 trials of 0.8 mA inescapable foot shocks with average 60 s intertrial interval (**Figure 36A**). No significant differences between the stressed (IS) and non-stressed (NS) animals after a 24 h interphase interval was detected (**Figure 36A**). Therefore, one more induction session was included in the second experimental design, which showed a trend towards increased number of escape failures in the 1st test that, however, did not reach significance. No differences between IS group (7.14 ± 1.461 attempts) and NS group (5.62 ± 1.500 attempts) could be detected in the 2nd test (**Figure 36B**). To increase the number of escape failures in the stressed animals, 180 instead of 120 inescapable foot shocks were delivered during the induction phase. Under these conditions, rats now showed significantly (repeated 2-way ANOVA, p=0.0342) increased escape failures in the 1st test (NS: 8.635 ± 2.490 attempts, IS: 18.901 ± 1.872 attempts) (repeated two-way ANOVA: time: F (1, 4) = 4.787, p=0.0939, treatment: F (1, 4) = 26.06, p=0.0070, time × treatment: F (1, 4) = 0.1915, p=0.6843), while no significant increases in the 2nd test (NS: 5.520 ± 1.500 attempts, IS: 13.426 ± 1.361 attempts) (**Figure 36C**). Finally, to assess the impact of the interphase interval on the development of depressive-like symptoms, a 48 h interval was introduced between the induction phase and testing phase. This change resulted in significantly increased escape failures in the IS group in 3 consecutive testing

sessions (T1: NS: (6.124 ± 1.861 attempts), IS: (22.500 ± 2.453 attempts), p=0.0180; T2: NS: (7.346 ± 3.114 attempts), IS: (23.450 ± 5.861 attempts), p=0.0207; T3: NS: (6.983 ± 2.104 attempts), p=0.0105) (repeated 2-way ANOVA: time: F (1, 6) = 0.06842, p=0.9346; treatment: F (1, 6) = 54.76, p=0.0003; time × treatment: F (2, 6)=0.1105, p=0.8971*)* (**Figure 36D**).

3.2.1 Number of induction sections, intertribal interval and number of shocks

	Induction	Number of sessions	Interphase interval (hrs)	Test session
A	IS (0.8mA, 60 trials) or NS	1	24	30 trials, fixed intertrial interval
B	IS (0.8mA, 60 trials) or NS	2	24	30 trials, fixed intertrial interval
C	IS (0.6mA, 90 trials) or NS	2	24	30 trials, fixed intertrial interval
D	IS (0.6mA, 90 trials) or NS	2	48	30 trials, scrambled intertrial interval

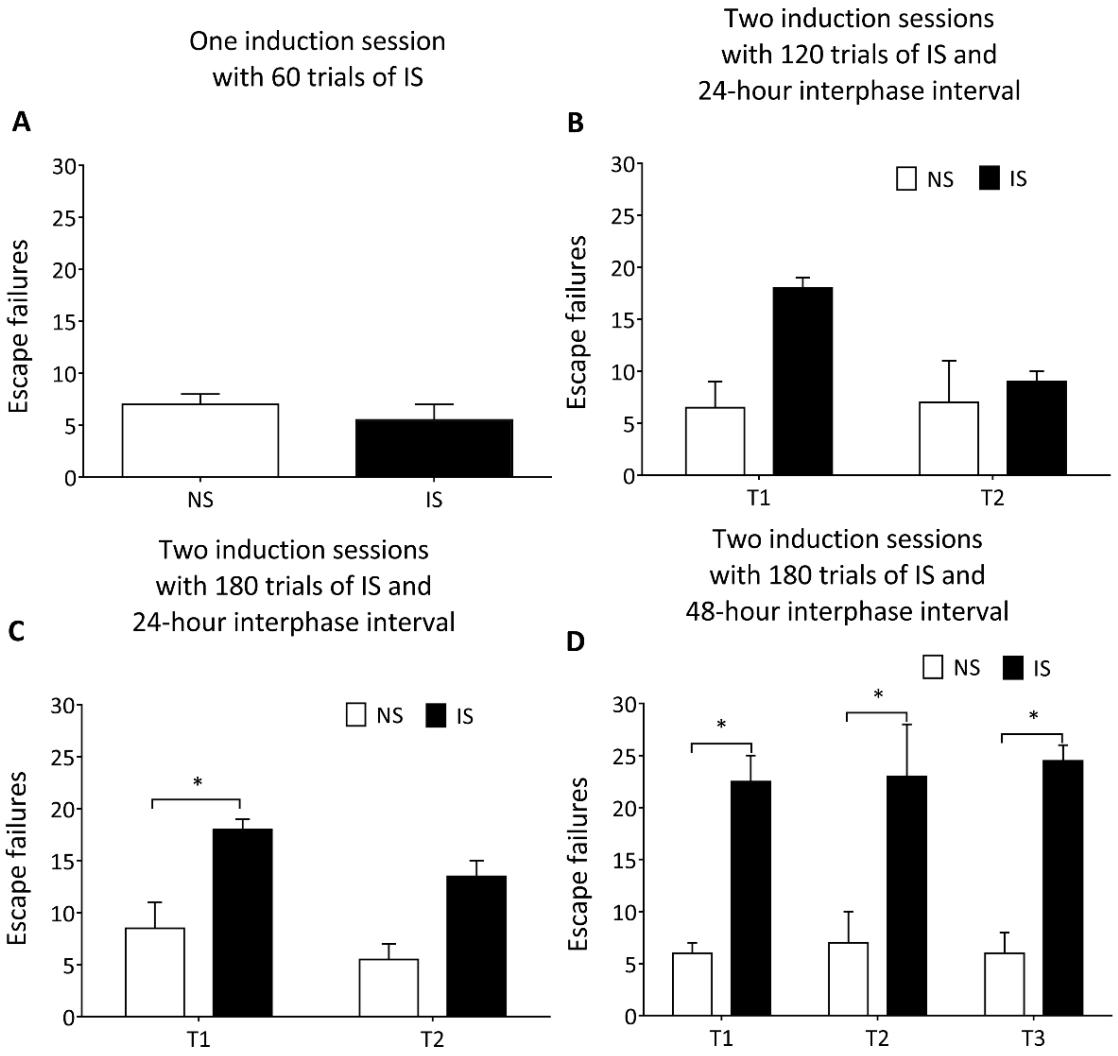

Figure 36. Optimisation of the learned helplessness (LH) model.
Three important parameters of the learned helplessness model: 1) numbers of induction sessions, 2) number of foot shocks and 3) the length of interphase interval were optimised in rats. Rats received either no foot shocks (NS, n=3) or inescapable foot shocks (IS, n=3) during the induction phase(s). Number of escape failures was measured in consecutive test sessions

(T) with 30 trials per session, after a variable length of interphase interval. The effect of one induction session on inducing depressive-like symptom was assessed (A), followed by assessment on two induction sessions (B). Different number of inescapable foot shocks were tested (C), prior to the optimisation on the length of interphase interval (D). *p<0.05 versus the NS group using repeated two-way Analysis of variance (ANOVA) followed by Sidak comparison tests. Error bar=SEM.

3.2.2 Different types of foot-shocks

To ensure the validity of IS in inducing depressive-like symptoms, ES were used as control to demonstrate that resulting symptoms were due to the inescapability of shocks rather than shocks *per se.* Rats exposed to IS exhibited significantly increased EFs in 3 consecutive tests (T1: (26.000 ± 1.000 attempts), T2: (24.200 ± 1.467 attempts), T3: (23.571 ± 1.131 attempts)), compared to NS group (T1: (7.000 ± 2.345 attempts), T2: (3.750 ± 1.436 attempts), T3: (6.750 ± 3.838 attempts)) (repeated 2-way ANOVA, time: F (2, 47) = 0.03207, p=0.9685, treatment: F (2, 47) = 44.50, p<0.0001, time × treatment: F (4, 47) = 0.8136, p=0.5229). As expected, no significant increases in the ES group were observed, compared to the NS group, it did not reach significance in comparison with NS group (**Figure 37B**).

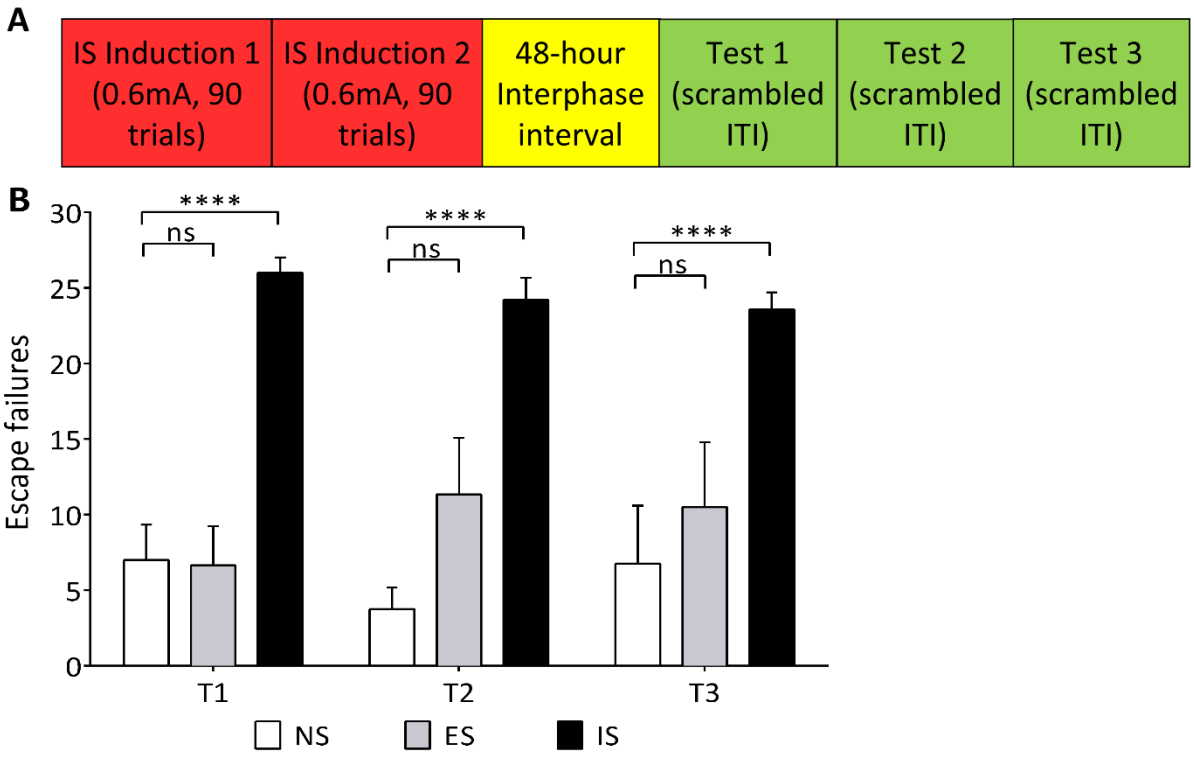

Figure 37. Effects of inescapable foot shocks on inducing depressive-like symptoms.
Rats exposed to either inescapable shocks (IS, n=8), escapable shocks (ES, n=6) and no shocks (NS, n=5) in the learned helplessness model with 2 induction training sessions. During the tests, the intervals between testing trials were set as 30 s ± 25 % (scrambled ITI) (A). The data is represented as the number of escape failures from three consecutive testing (T) sessions with a 24 h interval between sessions (B). ****p<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Error bar=SEM, ns=no significance.

3.3 Optimising the novel object recognition model

Memory loss has been frequently reported in the patients with depression (649). To investigate the effects of IS and novel drugs on depression-associated cognitive deficits, the NOR model was used, which is based on the tendency of rodents to spend more time exploring novel objects than familiar ones (650), while stressful situations usually impair object recognition (651). Briefly, the NOR model contains two phases: object learning and memory testing. In the learning phase, two identical objects are used while in the testing phase, one of the familiar objects is replaced with a novel object.

3.3.1 Persistence of natural memory in rats

To establish how long the animals memorize familiar objects in our laboratory setting, their natural memory was measured without stress exposure. In the first experiment, the animals spent (85.49 ± 6.300) % total object exploring time with the novel object after learning the familiar objects on 2 consecutive days, compared to the last day of test(repeated one-way ANOVA, time: $F(3, 6) = 4.043$, $p=0.0156$) (**Figure 38**).

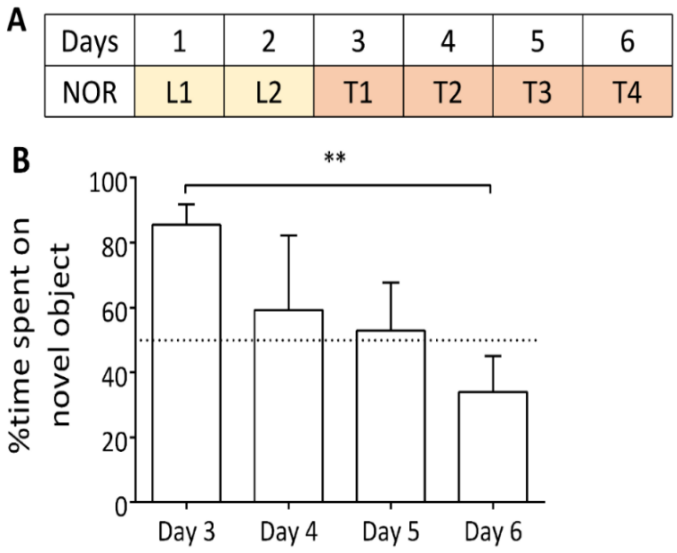

Figure 38. Persistence of natural recognition memory in rats. The natural recognition memory of rats was measured in the novel object recognition (NOR) model. Rats received 2 learning (L) sessions on days 1 and 2, followed by assessment of memory on 4 consecutive days (A). The data is represented as the percentage of time spent exploring novel objects to the total exploring time on both objects over a 5-minute period (B). **p<0.01 versus day 3 using repeated one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Error bar=SEM, n=3.

3.3.2 Effect of stress exposure on learning and memory

To evaluate effect of inescapable foot shocks on recognition memory in rats, in a second experiment, animals were exposed to 2 sessions of IS inductions (I) in the LH model, prior to memory learning session. Each IS induction session contained 90 trials of 0.8 mA inescapable foot shocks (**Figure 39A**). Object learning was conducted over a 15 min period, 10 min after the stress exposure. In the following memory test (24 h after the 2nd learning session), rats spent significantly more time exploring the novel object (familiar object: 28.60 ± 10.75 s, novel object: 82.4 ± 12.48 s, p=0.0309) (unpaired Student t test, t (4) = 3.266) (**Figure 39B**), suggesting that the foot shocks did not affect memory if the learning is conducted shortly after the stress exposure. This finding is in conflict with current clinical observations, where memory loss is reported in depressed patients (649).

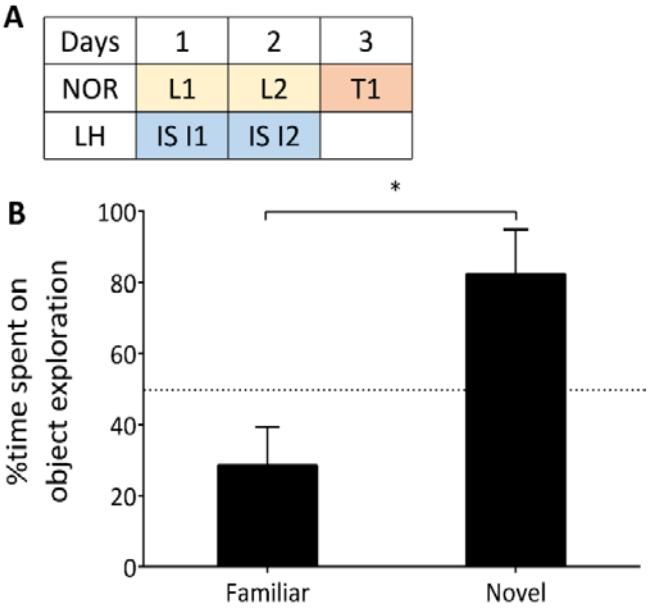

Figure 39. Effect of stress exposure on instant learning.
Rats received 2 inescapable-shock (IS) induction sessions in the learned helplessness (LH) model, 10 min before the object-learning (L) sessions in the novel object recognition (NOR) model on days 1 and 2. Memory of rats was measured in a single memory test over a 5 min period on day 3 (A). The data is represented as the percentage of time spent exploring familiar and novel objects respectively to total exploration time during test (B). *p<0.05 was calculated using unpaired Student t test. Error bar=SEM, n=3.

In a third experiment, object-learning sessions were carried out 24 h after the stress exposure. To parallel the LH and NOR tests, both learning sessions were conducted on the same day, during the interphase interval of the LH model. To ensure that rats learn familiar objects successfully, they received the 1st memory test 30 min after the 2nd object-learning session. In this scenario, no significant changes on % time spent on novel object exploration were observed in all testing sessions over a 5 min period (T1:73.64 ± 9.629 %, T2: 47.16 ± 22.484 %, T3: 44.16 ± 10.574 %) (multiple *t* test, T1: t (4) = 1.082, T2: t (4) = 0.1207, T3: t (4) = 2.061)(**Figure 40**).

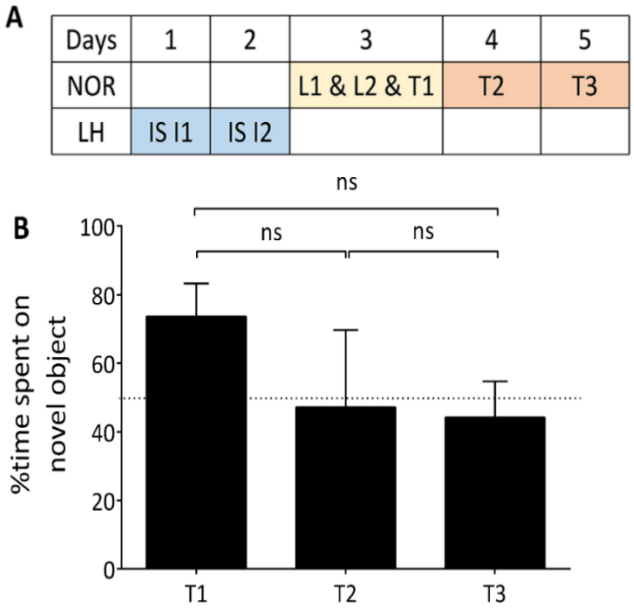

Figure 40. Effect of shock exposure on object learning and memory.
Rats were given 2 inescapable shock (IS) sessions in the learned helplessness (LH) model on days 1 and 2. Two object-learning (L) sessions of the novel object recognition (NOR) model were conducted 24 hours after IS exposure on day 3. There were 7 h interval between these two learning sessions. The 1st memory test (T1) was carried out 30 minutes after L2. T2 and T3 were conducted on days 4 and 5 respectively (A). The data represents the percentage of time spent exploring novel object to total object exploration time over a 5 min period (B). Statistical analysis was performed using multiple t tests with Holm-Sidak correction. Error bar=SEM, n=3, ns=no significance.

3.4 Optimising the fear extinction model

To further investigate the effects of stress and drug treatment on learning and memory of rats, the FE model was used, which requires active learning to dissociate a stress-induced fear from a stimulus (646). There are four phases in this model: habituation, FC or fear acquisition, FE learning and PE testing. In this experimental design, 2 different chambers were used. One was used for the habituation and FC phases, and the other was used for the FE training and PE tests. Illumination was only applied for the FE phase. In the FC phase, animals acquired fears to a sound signal through pairing the sound with a foot shock. Overall, no significant changes were observed across all phases (**Figure 41B-E**).

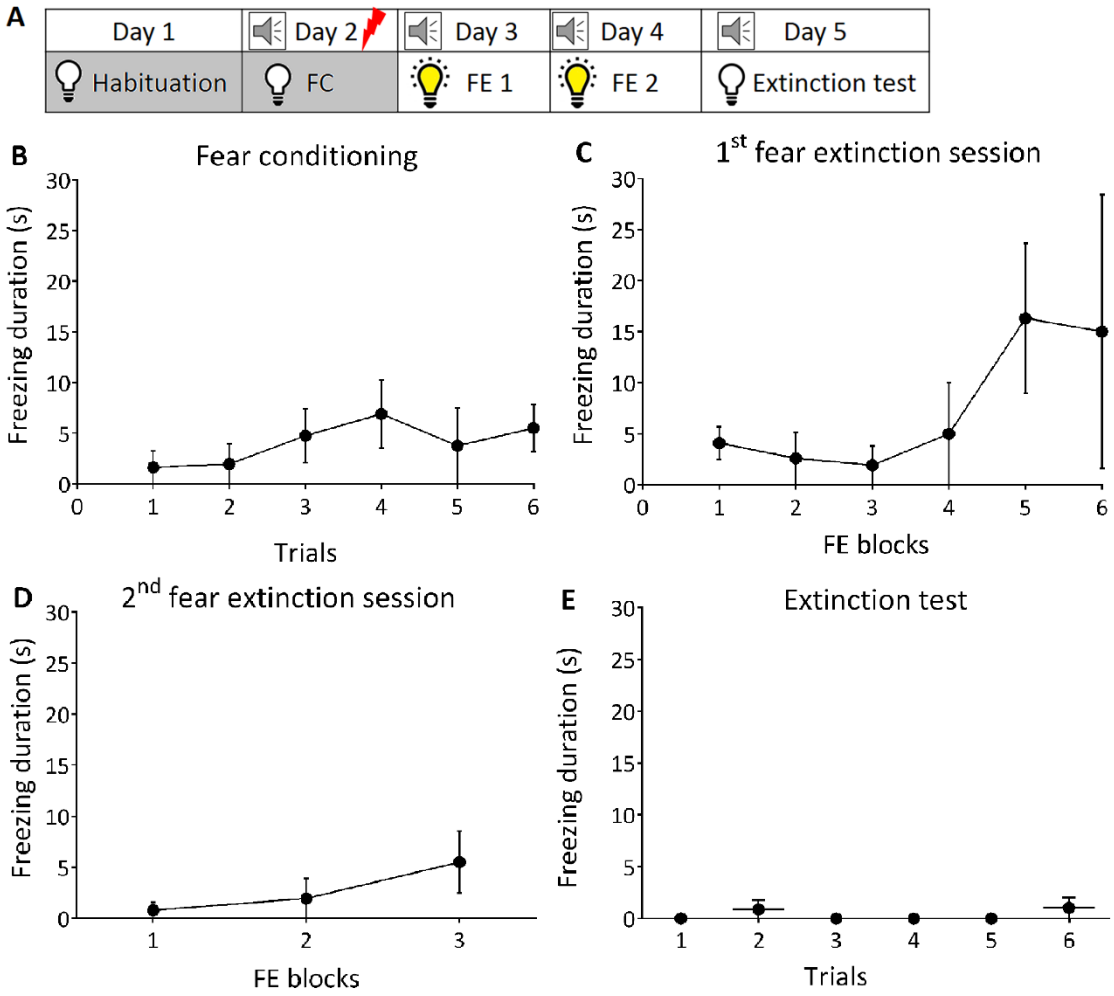

Figure 41. Optimisation of the fear extinction (FE) model.
The fear conditioning and extinction of rats were tested in the fear extinction (FE) model (A). Rats received fear conditioning (FC) training without illumination in the experimental arena on day 2 (B), followed by 2 sessions of FE training on days 3 and 4 respectively, where a different experimental chamber was used. In the FE phase, the chamber was illuminated with 15 Lux white light (C,D). The effect of extinction training was tested on day 5 (E). Duration of freezing during the sound signal was measured as an index of fear. The data is analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests, Error bar=SEM, n=4.

In the present study, multiple parameters of different pre-clinical behavioural models were measured as index of depression, stress perception, anxiety and cognitive dysfunction. The following content describes the effects of drugs in those models, which will be discussed based on the parameters of interest (**Table 6**).

Table 6. Parameters of interest in different behavioural models.
(LH: learned helplessness; EFs: escape failures; ITTs: intertrial interval transfers; ITI: intertrial interval; NOR: novel object recognition; OF: open field; TF: tail flick; HP: hot plate; FE: fear extinction)

Models	Measured parameters	Indication
LH	EFs	Depressive-like symptoms
	Number of avoidance	Avoidance behaviour
	Mean escape latency	Escape behaviour
	ITTs	Physical activity
	Freezing duration during ITI	Anxiety-like symptoms
NOR	Time spent on exploring objects	Recognition memory
OF	Moving duration	Physical activity
	%time spent in central area of OF chamber	Anxiety-like symptoms
	%distance travelled in central area of OF chamber	
	Rearing duration	
TF	Tail flick latency	Pain perception
HP	Hot plate latency	
FE	Freezing duration	Anxiety and learning

Chapter 4 The Psychopharmacological Effects of Morphine

4.1 Psychopharmacological effects of morphine in the learned helplessness model

4.1.1 Effect of morphine on depressive-like symptoms

After optimising the behavioural models to a stage where they produced reliable readouts, we evaluated the antidepressant-like effects of morphine in the LH model. Rats that were supplied by the UTAS animal breeding facility each week were allocated into control and/or treatment groups based on approach that was described in the section 2.3.4 of Chapter 2. The LH model was conducted in a two-compartment chamber, which was divided by a central door. Animals were allowed to transit between the two compartments freely. This model contained induction and testing phases. The induction phase was carried out with male SD rats on two consecutive days and included 180 trials of IS. After a 48 h interphase interval, the depressive-like symptoms of rats were assessed from days 4 to 6 by measuring the number of escape failures in each session (30 trials/session) (**Figure 42A**). Previously, morphine was reported to induce antidepressant-like effects in different pre-clinical models of depression (472,625,652). However, inconsistent results have been reported from different laboratories (525,653). Therefore, to exanimate the hypothesis of using opioids as antidepressants, and also to confirm the validity of the optimised LH model, morphine and the tricyclic antidepressant imipramine were tested. Drug treatment of rats started one hour after the 2nd IS induction training and subsequent injections were given from days 4 to 6 before the conduction of LH tests. The stressed saline-treated rats showed a significant increase in the number of escape failures across all tests (T1: 25.875 ± 1.125 attempts; T2: 24.250 ± 1.601 attempts; T3: 22.500 ± 1.452 attempts), compared to the NS group (T1: 9.200 ± 2.853 attempts; T2: 5.400 ± 1.990 attempts; T3: 5.400 ± 3.265 attempts) (repeated 2-way ANOVA, time: F (2, 47) = 0.03207, p=0.9685; treatment: F (2, 47) = 44.50, p<0.0001; time × treatment: F (4, 47) = 0.8136, p=0.5229), whereas both morphine (T1: 10.143 ± 2.098 attempts; T2: 5.857 ± 1.668 attempts; T3: 1.143 ± 0.986 attempts) and imipramine (T1: 8.500 ± 2.262 attempts; T2: 7.167 ± 2.725 attempts;

T3: 5.000 ± 1.789 attempts) significantly decreased IS-induced escape failures, compared to the IS group (repeated 2-way ANOVA, treatment: F (2, 57) = 104.3, p<0.0001) (**Figure 42B**).

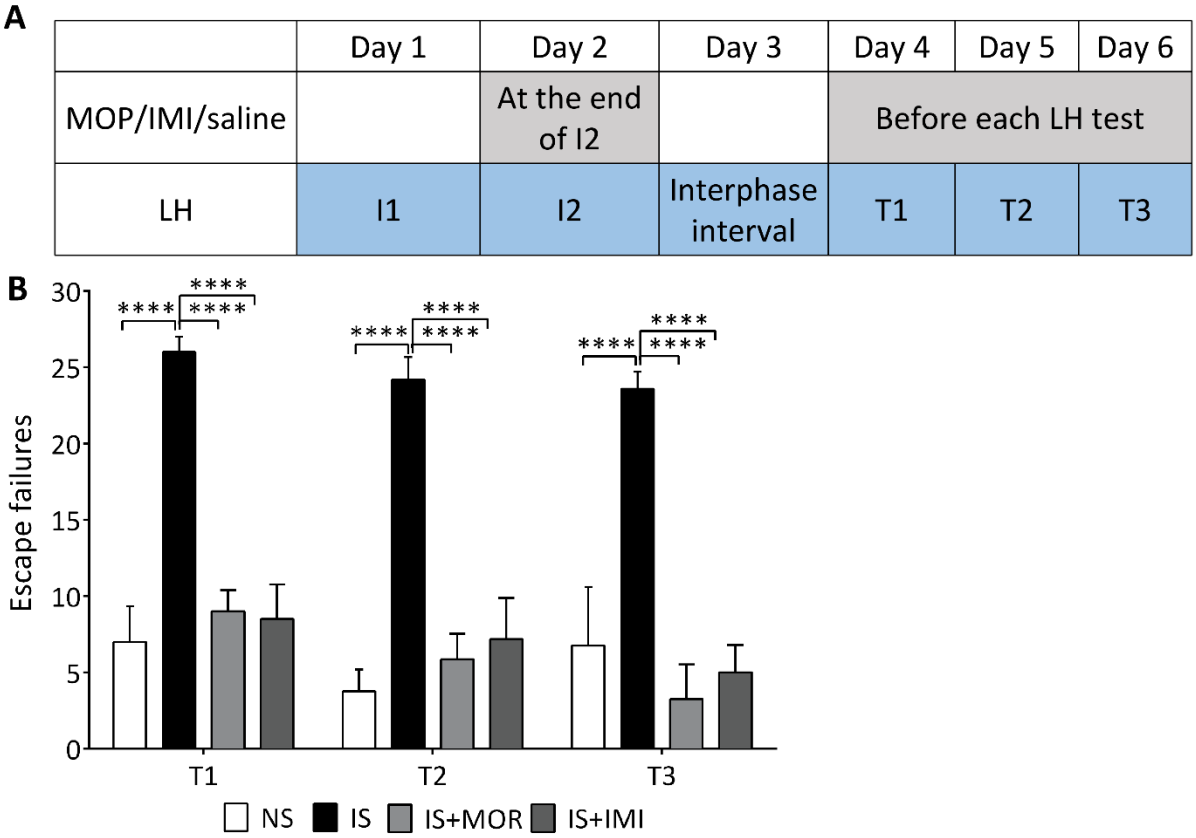

Figure 42. Effect of stress exposure and drug treatment on depressive-like symptoms. Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) induction training on day 1 and day 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and from days 4 to 6. The antidepressant-like effects of drugs were evaluated over 3 consecutive testing sessions from days 4 to 6, after a 48 h interphase interval between induction- and testing-phases (A). The data represents the number of escape failures over three consecutive testing sessions (T1-T3) (B). ****p<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

4.1.2 Effect of morphine on avoidance learning

In the induction phase of the LH model, animals learn that the outcome of foot-shocks is independent of their escape response. Therefore, the component of cognition is believed to be highly involved in the induction phase (654). In the testing phase, the animals' ability to learn could also enhance their escape performance. To investigate the involvement and potential

interference of cognition in our LH model, two different forms of learning: avoidance and escape learning, were assessed.

In the testing phase of the LH model, 30 pairs of signalled foot shocks were used. In each trial, a 3 s light signal was followed by a 3 s escapable foot shock. If rats transferred during the light signal to avoid the foot shock, the action was called avoidance. Even through exposure to IS can induce memory loss (**Figure 40**), the repeated testing pattern may still be learned by rats to avoid subsequent shocks, which eventually could result in false positive results in the treatment groups. Therefore, to evaluate if avoidance learning interfered the antidepressant-like effects of morphine and imipramine in our LH model, the number of avoidance events were measured. There were no significant differences in the 1st test between the groups. In the 2nd test, higher numbers of avoidance events were observed in all groups (**Figure 43**). Imipramine-treated rats exhibited significantly more avoidance behaviour (8.667 ± 2.728 attempts) in comparison with the IS group (3.125 ± 1.008 attempts) (repeated 2-way ANOVA, p=0.0321). In the 3rd test, no significant changes were observed between the NS (3.6000 ± 1.503 attempts), IS (3.250 ± 1.013 attempts) and imipramine-treated (7.333 ± 1.801 attempts) groups, whereas morphine-treated rats showed significantly more avoidance events (18.000 ± 2.526 attempts), compared to the IS groups (repeated 2-way ANOVA, time: F (2, 54) = 16.01, p<0.0001; treatment: F (2, 54) = 14.07, p<0.0001; time × treatment: F (4, 54) = 6.646, p = 0.0002).

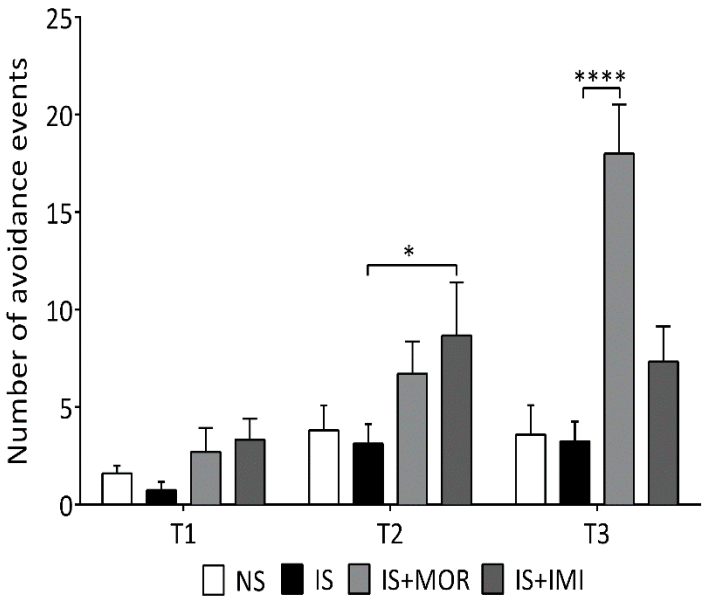

Figure 43. Effects of stress exposure and drug treatment on avoidance learning.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and from days 4 to 6 (for timeline see Figure 8A). The data represents the number of avoidance transitions over three consecutive testing sessions from days 4 to 6. *p<0.05 and ****p<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

4.1.3 Effect of morphine on escape learning

In the LH model, mean escape latency is measured as the time period from the start of a trial to the occurrence of transfer behavior. This parameter is used to quantify the level of escape learning involved during the testing trials. In essence, the faster the rats stop the trial, the better their memory is. Similar to avoidance learning, learning the escape pattern may also lead to false positive results in the treatment groups. In the 1st test, IS rats showed a significantly shorter mean escape latency 2.520 s (± 0.557) compared to the NS group 4.032 s (± 0.109) (repeated 2-way ANOVA, treatment: F (2, 54) = 11.31, p=0.0072), while no significance was observed in the morphine 3.453 s (± 0.069), p=0.5938 and imipramine 3.457 s (± 0.101), p=0.4409 groups. In the 2nd and 3rd test, no significant changes were observed between all groups. (**Figure 44**).

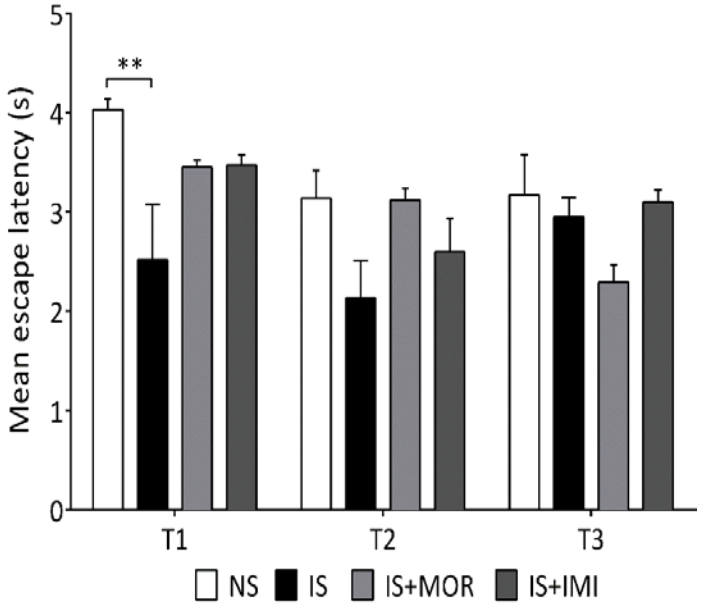

Figure 44. Effects of shock exposure and drug treatment on escape learning.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and from days 4 to 6 (for timeline see Figure 42A). The data represents the mean escape latency measured over three consecutive testing sessions from days 4 to 6. $^{**}p<0.01$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

4.1.4 Effect of morphine on physical activity

Because the number of escape failures are counted only when there are no transfers between two compartments of the LH chamber in each trial, the measurements highly rely on the physical activity of animals. In essence, the more active a rat behaves during the LH test, the higher its chance to randomly perform an escape behaviour. In the LH model, physical activity is represented as the number of transitions made during the intertrial intervals (ITTs) (429). Since morphine was reported to increase the locomotion in rats (655), it was important to assess if morphine-induced increased physical activity could be involved in its antidepressant-like effect. Generally, no significant changes were observed in the 1st and 2nd test. While in the 3rd test, morphine-treated rats showed significantly increased number of ITTs (15.286 ± 1.886),

compared to the IS group (7.125 ± 0.915) (repeated 2-way ANOVA, time: F (2, 54) = 5.219; treatment: F (2, 54) = 4.025; time × treatment: F (4, 54) = 5.219, p=0.0002) (**Figure 45**).

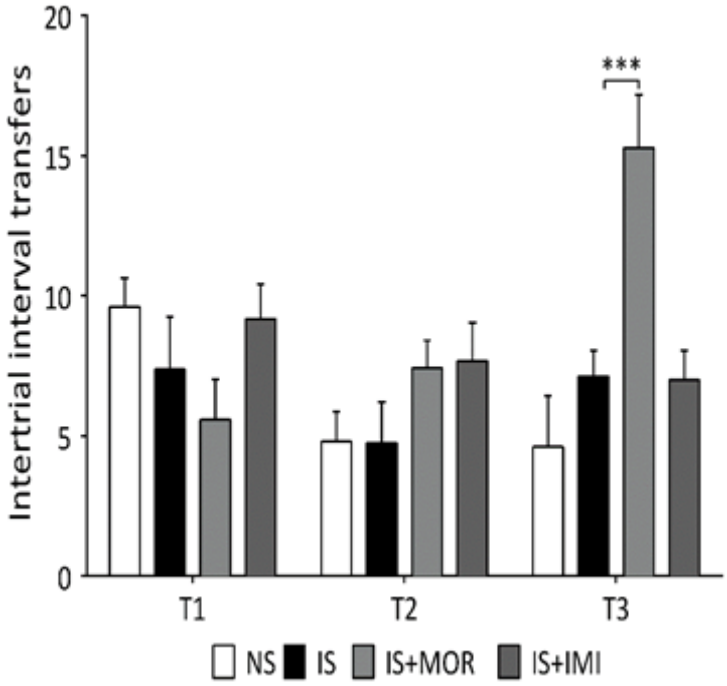

Figure 45. Effects of shock exposure and drug treatment on escape learning.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and from days 4 to 6 (for timeline see Figure 42A). The data represents the number of intertrial interval transfers (ITT) measured over three consecutive testing sessions from days 4 to 6. $^{***}p$<0.001 using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

4.1.5 Effect of avoidance learning, escape learning and physical activity on the antidepressant-like effect of morphine

Our previous results showed that morphine-treated animals showed significantly increased numbers of avoidance events (**Figure 43**) and ITTs (**Figure 44**). Based on the results, we hypothesised that the increased avoidance counts and ITTs may contribute to the observed escape events in morphine-treated rats. Therefore, in order to determine whether avoidance/escape learning and physical activity were at least partially responsible for the observed antidepressant-like effect of morphine, these parameters were correlated with the number of escape failures using residual plots. For regression analysis, residuals represent the difference between the observed value of the dependent variable and the predicted value (656). In my case, the dependent variables include the number of avoidance events, mean escape

latency and ITTs. The values of residuals in this study were calculated based on the line regression equations of those three factors against the escape failures in the morphine-treated group. Randomly scatted residuals in a residual plot indicate a linear relationship between two variables of interest. This would suggest that the change of one variable is likely to directly result in changes to the other variable. Thus the two variables of interest are highly correlated to each other. In my study, residuals randomly scattered in all plots (**Figure 46**). Hence, in supportive of the hypothesis, the avoidance/escape learning and physical activity of morphine-treated rats may partially be involved in or interfere with the observed escape performance of rats.

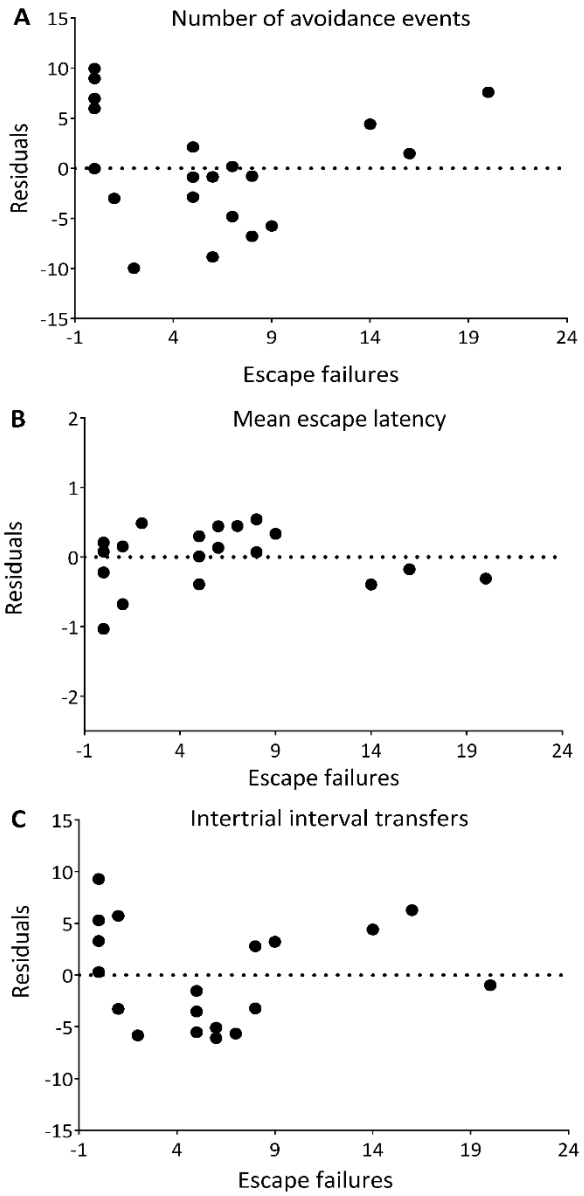

Figure 46. Correlation between avoidance learning, escape learning, physical activity and the antidepressant-like effect of morphine.
Correlations were generated using residual plots. The values used in the plots were calculated from all three LH tests of the morphine group (n=7). Residuals randomly scattered in all plots indicate some interference of learning and locomotion effects on the antidepressant-like effects of morphine in the LH model.

4.1.6 Effect of morphine on anxiety-like behaviour

It is well known that exposure to stress can result in the development of anxiety symptoms (657,658,659). Approximately 85 % of patients with depression also experience significant symptoms of anxiety. Similarly, comorbid depression occurs in up to 90 % of patients with anxiety disorders (660). Parallel to the clinical observations, exposure to IS over a short period can also induce anxiety-like symptom in animals (661). To access if the IS used in our LH model could produce anxiety-like symptoms as that observed in previous studies, freezing

duration, as surrogate marker for anxiety was measured during intertrial intervals in each testing session. In the NS and imipramine group, no significant changes were observed across 3 testing sessions. In the IS group, the freezing duration was significantly higher in the 2nd test (262.225 ± 41.548 s, p=0.0044), compared to the non-stressed rats (82.820 ± 21.387 s) (**Figure 47**). In contrast, significantly decreased freezing duration was observed in the morphine-treated group in the 2nd (and 3rd tests compared to the IS group (T2: 108.229 ± 36.004 s, p=0.0056, T3: IS + MOR: 17.714 ± 17.714 s, IS: 233.250 ± 29.620 s, p<0.0001) (repeated 2-way ANOVA, time: F (3, 66) = 2.954, p=0.0591, treatment: F (3, 66) = 12.12, p<0.0001, time × treatment: F (6, 66) = 1.978, p=0.0813),.

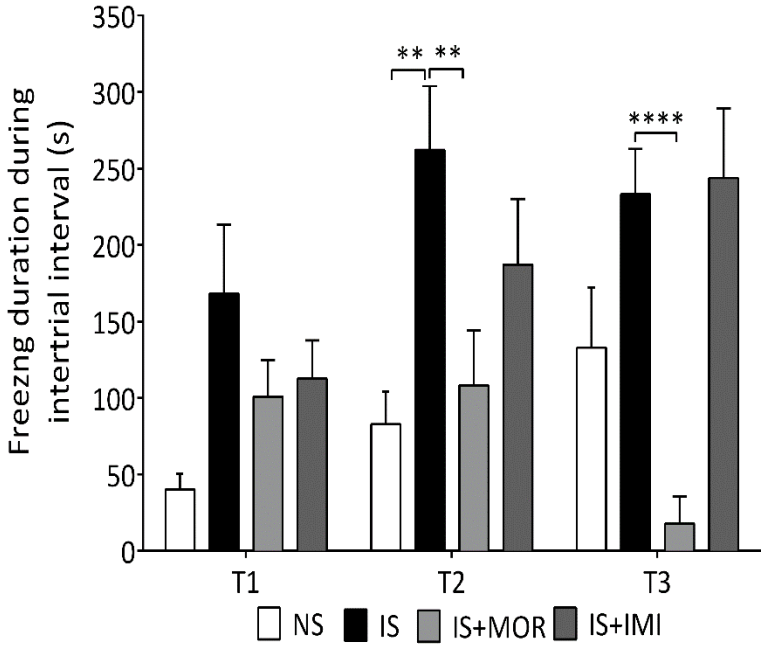

Figure 47. Effects of prior shock exposure and morphine on anxiety-like behaviour.
Saline-treated rats were either unstressed (NS, n=5) or exposed to inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and from days 4 to 6 (for timeline see Figure 42A). The data represents the freezing duration measured over 3 consecutive testing sessions from days 4 to 6. **p<0.01 and ****p<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

4.1.7 Effect of shocks and morphine on body weight

Weight loss after exposure to shocks in the LH model has been reported previously (662). However, both weight loss (663) and weight gain (664) were observed in depressed patient resulting from personal eating habit and attitudes, suggesting a complex relationship between depression and weight changes in clinic. Therefore, to evaluate the influence of stress and

helpless emotional status on body weight in my experiment, animals were weighed over the entire duration of the LH procedure (**Figure 48A**). In all groups, no body weight changes were detected in all groups (**Figure 48B-E**). Due to the significant body weight differences of the animals and the attempt to use a weight based randomization scheme, the groups nevertheless showed different average body weights.

A

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MOP/IMI/saline		At the end of I2		Before each LH test		
LH	Induction 1	Induction 2	Interphase interval	T1	T2	T3
Body weight measurement	Basal, before the exp.	Before the exp.		Before the exp.		

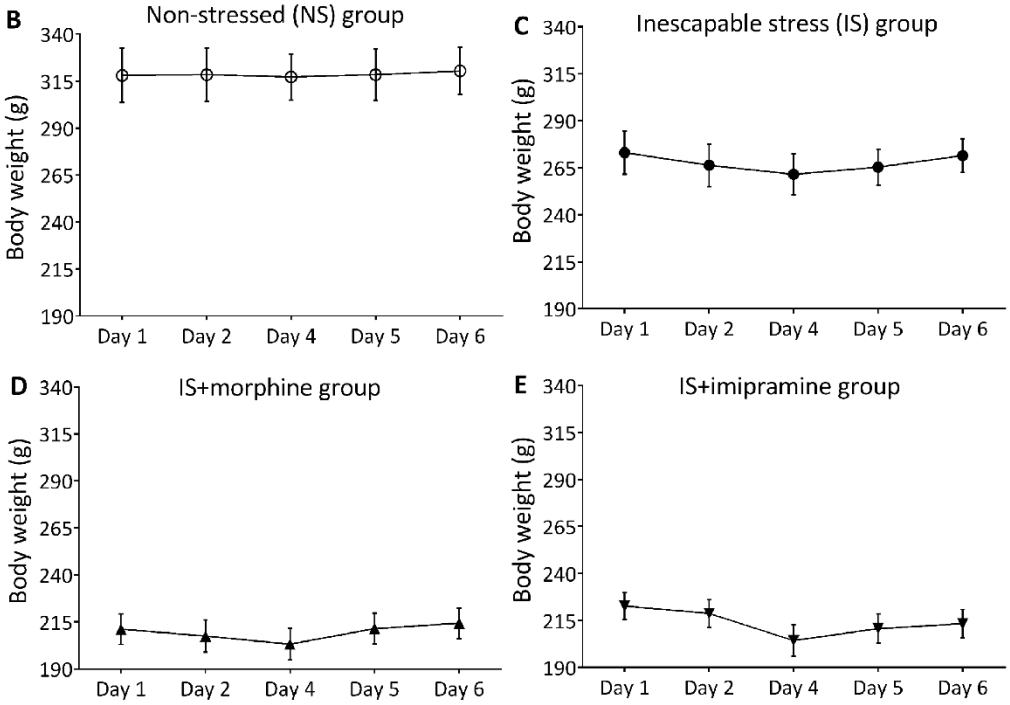

Figure 48. Effects of shocks and treatment on body weight changes.
Body weight changes of rats were measured throughout the experiment except on day 3 (A). Saline-treated rats received either no stress (B, NS, n=5) or inescapable stress (C, IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (D, MOR, 5 mg/kg/day, n=7) or imipramine (E, IMI, 50 mg/kg/day, n=6) on day 2 and days 4-6. Statistics were performed versus day 1 within same groups using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests, Error bar=SEM.

To compare the influence of foot-shocks and treatments on body weight, the percentage changes in body weight were calculated (**Figure 49**). In both control groups, no significant %

changes against the starting body weight on day 1 were detected (**Figure 49A, B**). However, in the morphine-treated group, significantly increased body weights were detected on day 5 and day 6 (Day 1: -2.189 ± 0.796; Day 5: 1.927 ± 0.526, p=0.0062; Day 6: 1.057 ± 0.989, p=0.043) (repeated 2-way ANOVA, time: F (12, 100) = 2.157, p=0.0195, treatment: F (4, 100) = 15.06, p<0.0001, time × treatment: F (3, 100) = 10.05, p<0.0001) (**Figure 49C**). In the imipramine-treated group, a significant decrease in body weight on day 2 was observed (repeated 2-way ANOVA, Day 1: -1.353 ± 0.624; Day 2: -8.499 ± 1.302, p=0.00006) (**Figure 49D**).

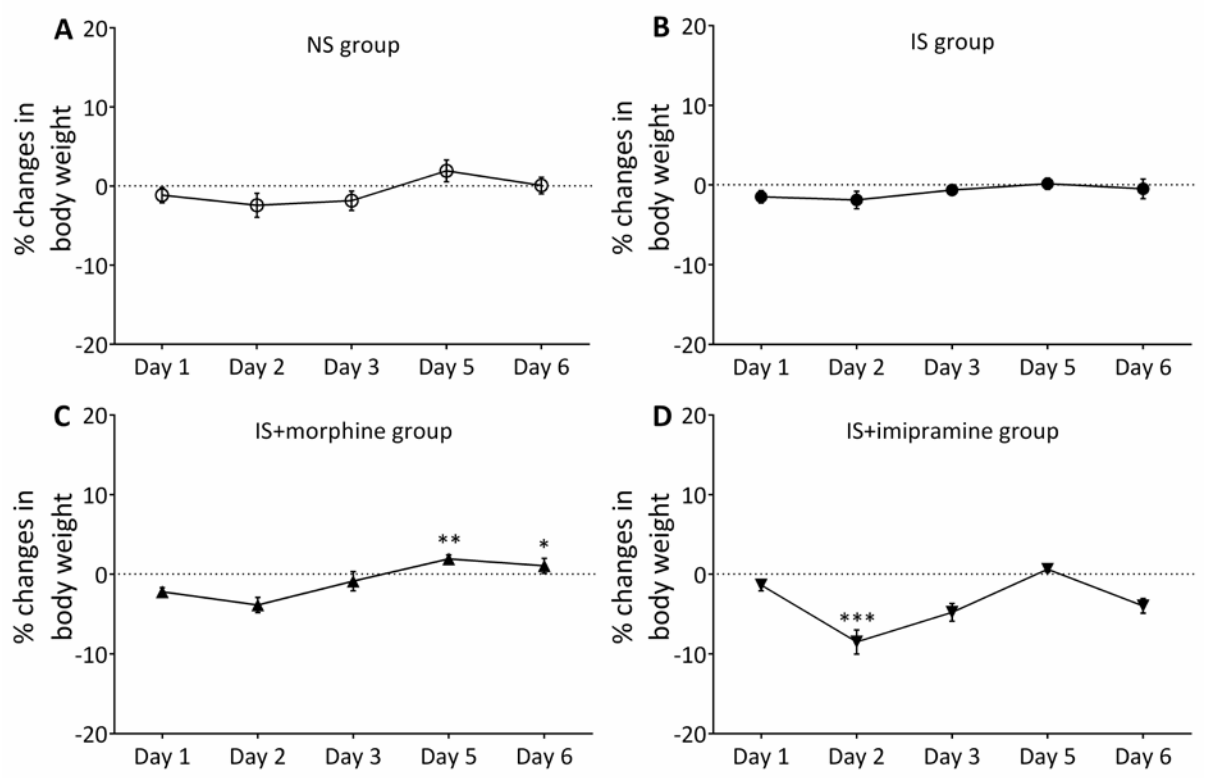

Figure 49. Percentage changes in rat body weight.
The body weights of rats were measured throughout the experiment except on day 3 (Figure 48A). Saline-treated rats received either no stress (B, NS, n=5) or inescapable stress (C, IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (D, MOR, 5 mg/kg/day, n=7) or imipramine (E, IMI, 50 mg/kg/day, n=6) on day 2 and days 4-6. The percentage changes of body weight in each group were calculated based on the starting body weight measured on day 1. Statistics were performed versus day 1 within same groups. *p<0.05, **p<0.01 and ***p<0.001 using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests.

4.2 Psychopharmacological effects of morphine in the open field test

4.2.1 Effect of morphine on physical activity

The OF test is widely used to measure general physical activity and anxiety in rodents (617). To investigate the impact of IS and treatment on physical activity and anxiety-like symptoms in more detail, several parameters (1. time spent on moving in the OF chamber over 5 min test (=moving duration), 2. percentage time spent and distance travelled in the centre of the OF chamber, and 3. the number of rearing) were measured. In our model, the OF test was carried out on the same days as the LH procedure. OF tests of controls were measured on days 1, 2 and 6, while OF tests of the morphine group were also performed on days 4 and 5 (Figure 50A). Generally, no changes on total moving duration were detected between IS and NS groups across 3 testing sessions (Figure 50B). In the imipramine-treated group, a significant decrease in moving duration was observed on day 6, compared to the IS groups (IS: 161.667 ± 18.828 s, IS+IMI: 156.400 ± 13.670 s, p=0.0041) (repeated 2-way ANOVA, time: F (2, 36) = 0.8038, p=0.4555, treatment: F (2, 18) = 5.736, p=0.0118, time × treatment: F (4, 36) = 7.731, p=0.0001) (Figure 50B). Morphine-treated rats, on the other hand, showed significant increased moving duration from day 4 onwards, compared to the basal levels measured on day 1 (basal: 147.200 ± 5.416 s, day 4: 198.800 ± 14.491 s, p=0.00054, day 5: 199.667 ± 19.989 s,

p=0.0034, day 6: 189.700 ± 6.756 s, p=0.043) (repeated 1-way ANOVA, treatment: F (4, 25) = 7.515, p=0.0004) (Figure 50C).

A

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MOP/IMI/saline		At the end of I2		Before each LH test		
LH	Induction 1	Induction 2	Interphase interval	T1	T2	T3
OF tests of MOP	Basal, before induction 1			After treatment and before each LH test		
OF tests of control	Basal, before induction 1					Before the LH test

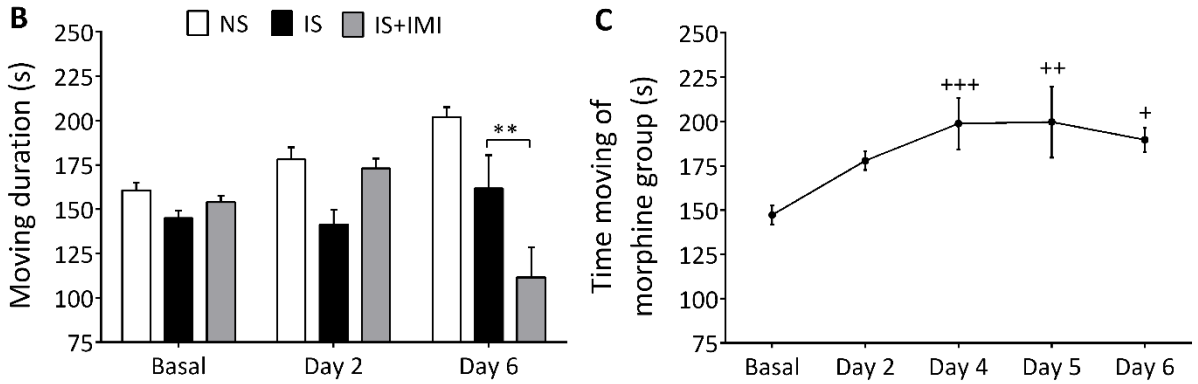

Figure 50. Effect of drug treatment on physical activity.
The physical activity of rats was measured in the open field model. Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) or imipramine (IMI, 50 mg/kg/day, n=6) on day 2 and days 4-6 (A). Time spent on moving of MOR group was measured throughout the experiment except day 3 (C), while moving duration of reminding groups was measured on days 1, 2 and 6 (B). The data represents total moving time over a 5 min period. $^{**}p<0.01$ using repeated two-way Analysis of variance (ANOVA) followed by Tukey comparison tests. $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus basal level in the MOR group using repeated one-way analysis of variance (ANOVA) followed by Dunnett comparison tests. Error bar=SEM.

4.2.2 Effect of morphine on anxiety-like symptoms

The OF test was originally described as “a test of emotionality” (665). In this test, animals are subjected to an inescapable open chamber and their activities including rearing, moving, jumping and resting are recorded (665). When compared to naïve animals, anxious rats tend to

avoid entering into the central area of the testing chamber (666). In addition, activity of rearing has been used as an index of exploratory behavior in the OF test and reported to be reliable to indicate stress levels of animals (617). Three parameters, 1) percentage time spent in the central area of the OF chamber, 2) percentage of distance the rats travelled in the centre and 3) the duration of rearing are used as indicators of anxiety-like behaviour. In our study, no significant changes for time (Figure 51A, D), distance travelled in the central area of the OF chamber (Figure 51B, E) or rearing behaviour (Figure 51C, F) were observed for all groups overall.

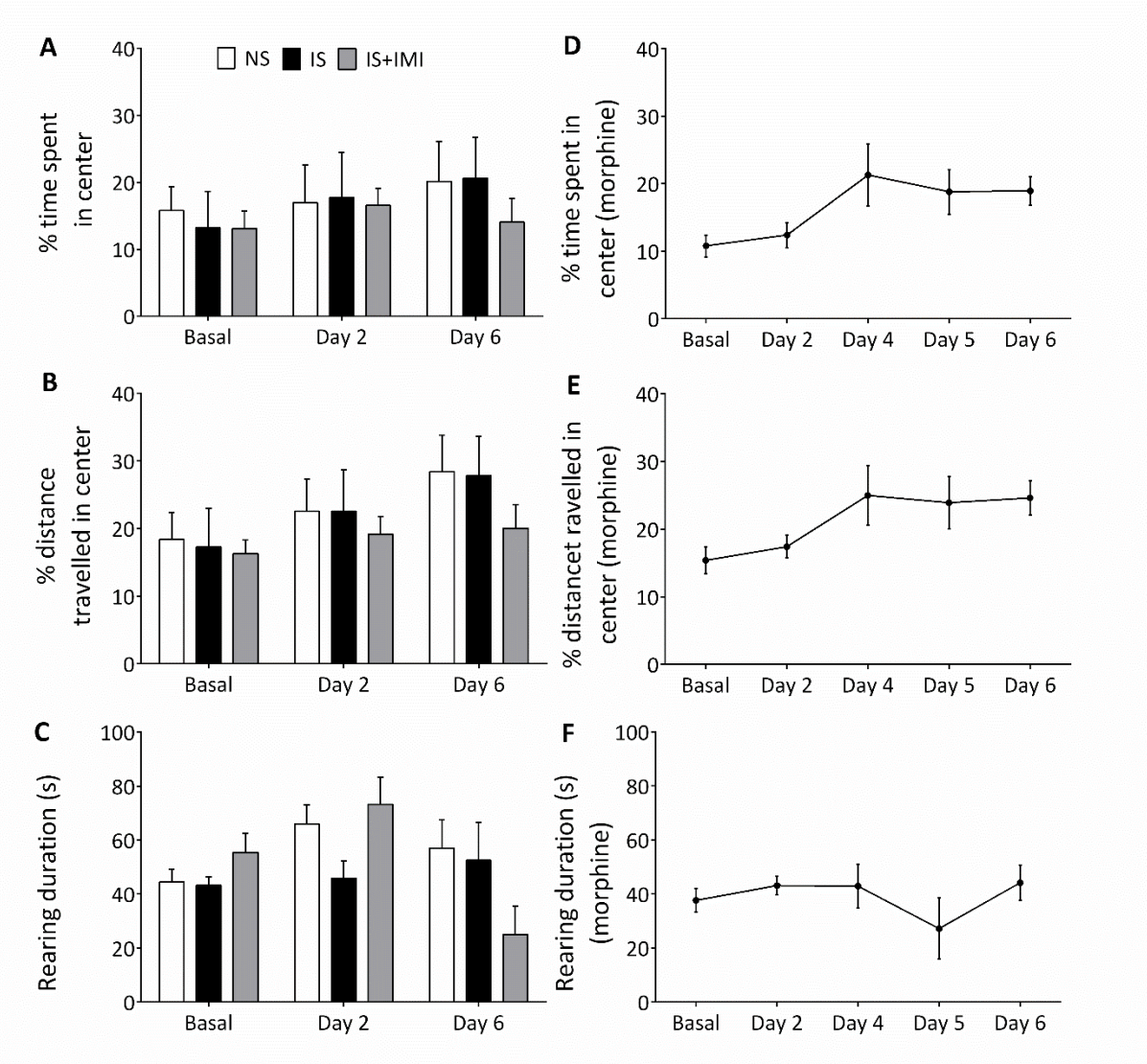

Figure 51. Effects of drug treatment on anxiety-like behaviour.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with morphine (E-F; MOR, 5 mg/kg/day, n=7) or imipramine (A-C; IMI, 50 mg/kg/day, n=6) on day 2 and days 4-6 (for timeline see Figure 49A). The percentage of time (A, D), percentage of distance (B, E) and duration of rearing (C, F) were

measured over 5-minute period in the open field model. Statistical analysis used repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests; Error bar=SEM.

4.3 Psychopharmacological effect of morphine in the light and dark model

As above stated, exposure to stress can induces anxiety or anxiety-like symptoms in both humans and rodents (667). In order to evaluate the specificity of our LH model to induce depressive-like symptoms, the LD model for anxiety was used. The measurement of anxiety-like symptoms was carried out on days 1, 2 and 6 before the LH procedure. The time spent in the dark compartment of the LD chamber over a 5-min test was measured as an indicator of anxiety-like symptoms. No significant changes on time spent in the dark compartment was observed in the IS group. On contrast, a significant decrease was detected in the 3rd LD test in the morphine group, compared to the 1st test (repeated 2-way ANOVA, p=0.0333) (**Figure 52B**).

A

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MOP/saline		At the end of I2		Before each LH test		
LH	Induction 1 (I1)	Induction 2 (I2)	Interphase interval	T1	T2	T3
LD	Before the LH exp. (basal)	Before the LH exp.				Before the LH exp.

B

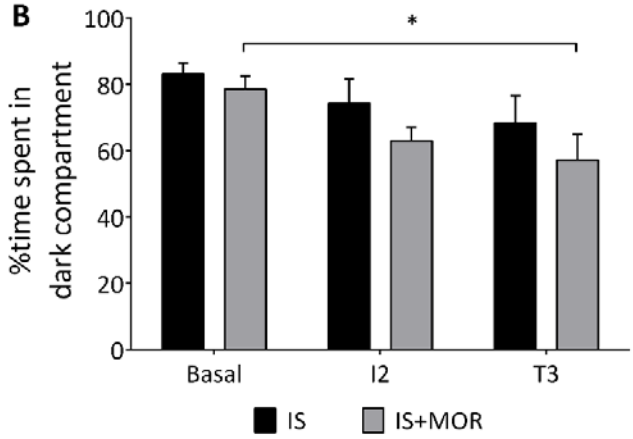

Figure 52. Effects of stress exposure and morphine on the induction of anxiety-like behaviour.

Anxiety-like symptoms of rats were measured in the light and dark (LD) model. Saline-treated rats were exposed to inescapable stress (IS, n=6) on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) on day 2 and days 4-6 (A). The data presents the percentage of time that rats spent in the dark compartment of the LD chamber and measured on days 1, 2 and 6 (B). $^{*}p<0.05$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests, Error bar=SEM.

4.4 Effects of shocks and morphine on nociception

Stress-induced analgesia (SIA) is a natural pain suppression response when mammals are exposed to aversive stressful events (668). Exposure to IS in the LH model was reported to induce opioidergic system-mediated SIA (669). A physiological decrease in pain perception could conceivably decrease the animals' sensation and fear to the foot shocks, which would reduce escape failures in the LH testing sessions. To determine if the analgesic effect of morphine could interfere with its antidepressant-like effects, the tail-flick (TF) test and hot-plate (HP) test were used. Both tests were performed before the LH procedure. In the TF test, the tail flick latency of stressed saline-treated rats significantly increased on day 2 after exposure to 90 IS (basal: 4.367 ± 0.170 s, day 2: 5.206 ± 0.449 s, p=0.0398), while the TF latency returned to basal levels on day 6 (3.128 ± 0.339 s, p=0.0006) (repeated 2-way ANOVA, time: F (2, 22) = 4.628, p=0.0210; treatment: F (1, 11) = 0.4875, p=0.4995; time × treatment: F (2, 22) = 6.026, p=0.0082). In this test, pain perception of morphine-treated rats remained the same in all tests (**Figure 53B**). In the HP test, no significant differences were detected either between or within groups across the three tests (**Figure 53C**).

A

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
MOP/saline		At the end of I2		Before each LH test		
LH	Induction 1	Induction 2	Interphase interval	T1	T2	T3
TF and HP	Basal, before the LH exp.	Before the LH exp.				Before the LH exp.

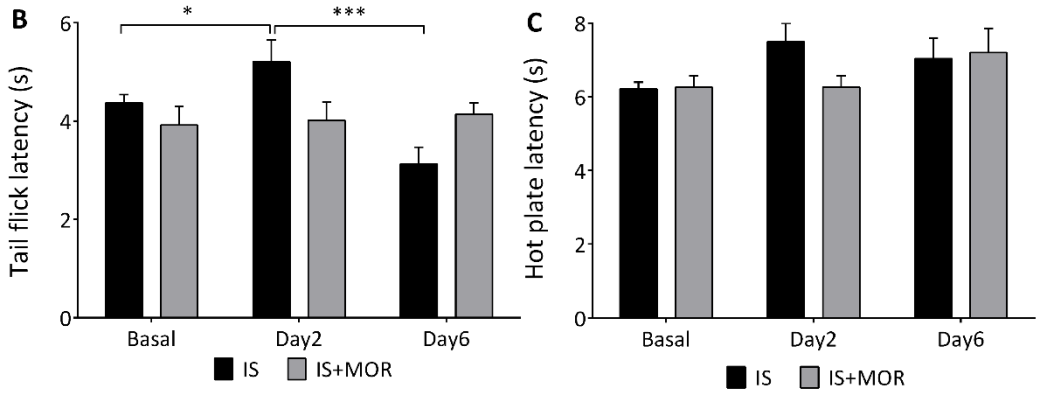

Figure 53. Effects of stress exposure and morphine on pain perception.
Saline-treated rats were exposed to inescapable stress (IS, n=6) on days 1 and 2. Stressed rats were treated with morphine (MOR, 5 mg/kg/day, n=7) on day 2 and days 4-6. Pain perception was measured on days 1, 2 and 6 (A) in the tail flick (TF) test (B) and the hot plate (HP) test (C). $^{*}p<0.05$ and $^{***}p<0.001$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests, Error bar=SEM.

Chapter 5 The Psychopharmacological Effects of Bifunctional Opioids

5.1 Toxicity of 1001 and 1003 *in vitro*

Activation on a single receptor often produces insufficient biological activity in medical practice or is associated with adverse effects (670,671,672). Therefore, recent research focused on ligands that display multiple specificities and/or activities (673). Bi- or multifunctional drugs promise to display significantly improved potency due to synergistic effects and might produce less side effects than compounds acting at a single target (368). So far, novel DOP agonists have demonstrated both anxiolytic- and antidepressant-like effects in animal models. For example, UFP-512 decreased immobilization duration in the FST (a model of depression), as well as evoked more open arm visits in the elevated plus maze (a model of anxiety), which could be fully blocked by the selective DOP antagonists naltrindole (512). Similarly, another full DOP agonist SCN80 exhibited positive therapeutical effects in multiple pre-clinical models of anxiety and depression (674). In addition, there is some evidence that the DOP receptor can modulate the function of the MOP receptor. Pharmacological inhibition of DOP receptor activity was reported to enhance the binding and signaling of MOP receptor agonist *in vitro* (675,676). This functional interaction of both receptors is also supported by *in vivo* results that described that the DOP receptor agonists DPDPE was able to induce mild analgesic effects, partially by activating the MOP receptor (677). On the other hand, chronic morphine administration increased the expression of DOP receptors in the rat spinal cord (678). Finally, morphine tolerance was not observed in DOP knockout mice (355), which strongly suggests a possible role for the DOP receptor in regulating morphine-mediated tolerance. . Therefore, the rationale to develop bifunctional opioid agonists for MOP and DOP receptors is to develop novel drugs that can produce both antidepressant and analgesic effects at same time, while harbouring fewer adverse effects.

Both novel bifunctional opioids 1001 (also called UFP505) and 1003 were synthesised in cooperated with the School of Chemistry. Both purity of 1001 and 1003 was assessed by our collaborators (Department of Chemistry, University of Tasmania) using standard analytical techniques (LC-MS/MS, NMR) and the compounds used in this study always showed a purity of above 98%. Based on a previous report (679) and so far unpublished data (manuscript in preparation) derived from another PhD student at UTAS (A. Paul), the bifunctional opioid 1001 (**Figure 54A**) simultaneously activates the MOP receptor and blocks the DOP receptor. In contrast, the closely related compound 1003 (**Figure 54B**) simultaneously activates both MOP and DOP receptors.

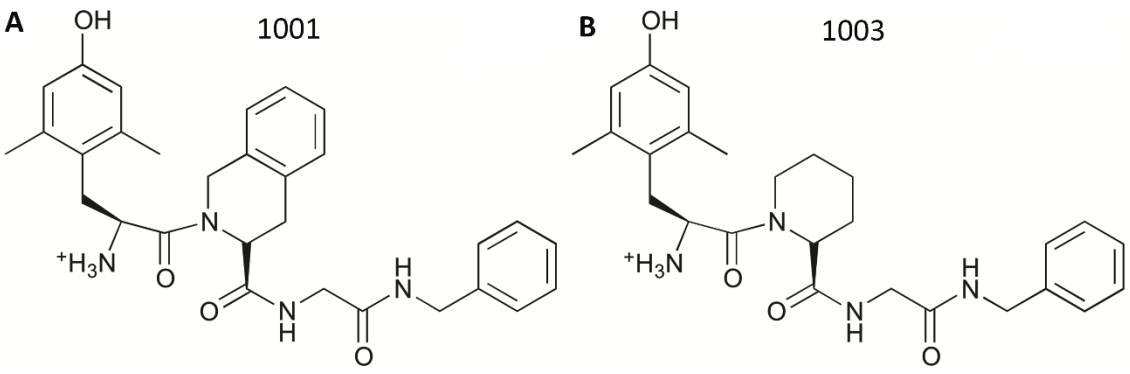

Figure 54. Chemical structures of 1001 and 1003.
Both novel bifunctional opioids show selectivity for both mu-opioid (MOP) and delta-opioid (DOP) receptors but display different activities.

Since morphine is a potent MOP agonist with a range of behavioural effects in our models, we hypothesized that 1001 and 1003 could also show potential behavioural effects in the same model. However, before testing this possibility *in vivo* we assessed the safety profiles of 1001 and 1003 *in vitro.* The toxicity of both novel opioids was tested with regards to cellular bioenergetics and proliferation. For this purpose, we assessed cellular ATP levels and protein content in the hepatocarcinoma cell line (HepG2) in the absence and presence of these novel compounds. Typically for small molecular drugs, the highest concentrations are observed in the liver. Therefore HepG2 cells were selected as a commonly used cell culture test system to

predict potential liver toxicity *in vivo*. The cells are well characterized and highly sensitive to different kinds of xenobiotics and cytotoxic compounds and are therefore routinely used in drug discovery (680,681). In addition, cellular ATP levels were also standardised on protein content and drug effects were expressed as a percentage of untreated control. Significantly increased ATP levels/mg protein were observed in morphine-treated cells after 6 hours (untreated cells: 105.21 ± 7.36 %, morphine-treated cells: 120.90 ± 9.87 %; p=0.0170), compared to untreated cells (**Figure 55A**). After both 12 and 24 hour incubations, 1003-treated cells showed significant increased ATP levels (12-hour: untreated cells: 115.64 ± 6.82 %, 1003-treated cells: 138.79 ± 10.86 %, p=0.0001; 24-hour: untreated cells: 131.22 ± 11.51 %, 1003-treated cells: 173.96 ± 7.06 %, p<0.0001). In contrast, after 24 hour incubation a significant drop of ATP/mg protein content was detected in the 1001-treated cells (1001-treated cells: 81.36 ± 9.55 %) (2-way ANOVA, time: F (4, 80) = 49.97, p<0.0001; treatment: F (3, 80) = 30.54, p<0.0001; time × treatment: F (12, 80) = 19.59, p<0.0001) (**Figure 55A**). A significant increased protein content was observed after 24 hours of incubation in the 1003-treated cells, compared to the untreated cells (untreated cells: 106.23 %, 1001-treated cells: 62.14 %, 1003-treated cells: 127.86 %). However, the opposite was seen in the 1001-treated cells (2-way ANOVA, time: F (4, 80) = 4.789, p=0.0243; treatment: F (3, 80) = 9.375, p=0.00626; time × treatment: F (12, 80) = 3.549, p=0.0361) (**Figure 55B**).

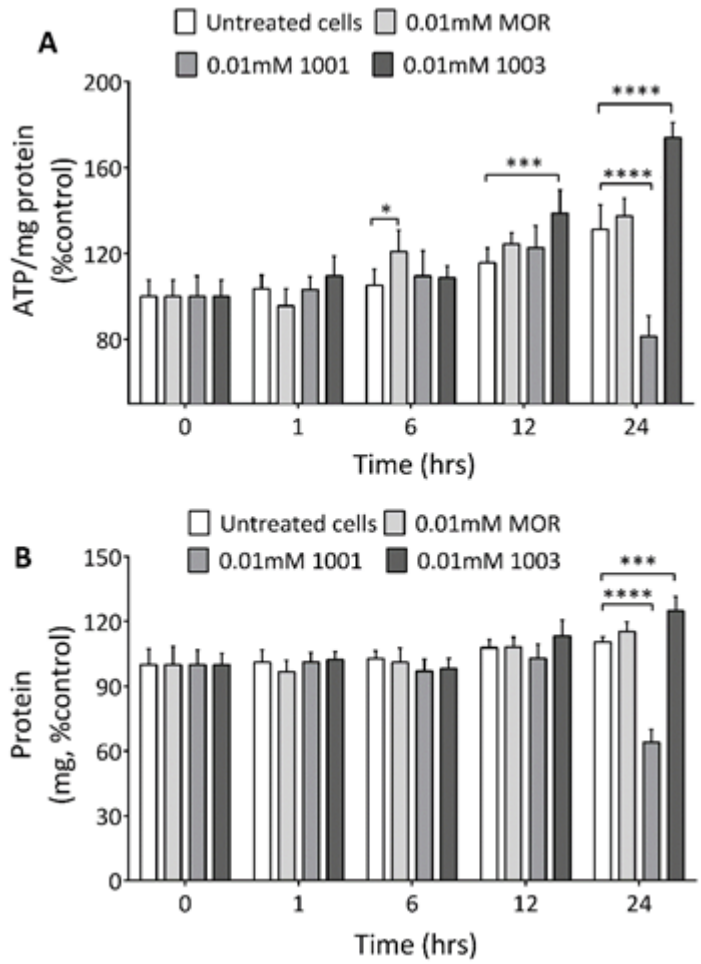

Figure 55. Effects of 1001 and 1003 on cell viability.
Cell variability was measured using liver hepatocellular carcinoma (HepG2) cells. Cells were incubated with 0.01 mM of morphine (MOR), 1001 and 1003 respectively for different period (0 – 24 h). Toxicity was assessed as ATP/mg protein (A) and protein content (B) in the lysate (mg/ml). The data represents the average of three independent experiments and is expressed as percentage of the untreated control. $^{*}p<0.05$, $^{***}p<0.001$ and $^{****}p<0.0001$ using two-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. Error bar=SD, n=6.

5.2 Psychopharmacological effects of 1001 and 1003

5.2.1 Psychopharmacological effects of novel opioids in the learned helplessness model

5.2.1.1 Effects of 1001 and 1003 on depressive-like symptoms

UFP505 (1001) exhibits antinociceptive effects in rats only after intrathecal injection which suggests that brain penetration of subcutaneous injected drug is poor (682). Therefore, one of the major aims of the present research program was to improve on the limited uptake of 1001 into the brain. To achieve this goal, several analogues were synthesized by the Department of Chemistry (UTAS) that were modified to improve brain penetration. One of those 1001 analogues, termed 1003, surprisingly showed DOP receptor agonist activity (PhD thesis A. Paul, see Appendix 2 for details) in contrast to 1001, which is a DOP receptor antagonist. Since

there is some evidence that DOP receptor agonists can be effective in treating mood disorders (289), it was hypothesized that 1003 might also provide beneficial antidepressant-like effects in our model. In addition, functional antinociceptive experiments suggested that 1003 had improved brain permeability compared to 1001 (PhD thesis A. Paul, see Appendix 3 for details). This study, evaluated the differential behavioural effects of 1001 and its analogue 1003, where 1001 was employed as a control compound that was expected to be inactive. Overall, my results provide some evidence that the structure of 1001 can be successfully modified to improve its pharmacological properties.

Our *in vitro* results suggest that 1003 is safe to be used in animal studies. Even though 1001 reduced the ATP/mg protein levels of cells over 24 h incubation period, its *in vivo* safety in rats has been evaluated in a previous study (671). To compare the effects of 1001 and 1003 on animal behaviour against morphine treatment, both bifunctional opioids were tested in the same animal models as described above. In the experiment, rats that were supplied by the UTAS animal breeding facility each week were allocated into the different treatment groups using a completely radomised method as previously described (634).

For this purpose, identical dose and injection regimes to the ones used with morphine were also employed for 1001 and 1003. In this experiment, no contemporaneous controls were used in parallel with the treatment groups for three reasons. Firstly, the LH paradigm is a very robust model, since consistent control and treatement results were even obtained from experiments that were performed in different calendar years (**Table 8 and 9 in Appendix**). Secondly, due to the technically difficult nature of the integrated testing paradigm, it was impossible to test more than 4 rats per day without added interfering factors such as circadian effects and added environmental stress. Thus, to add simultaneous testing of control animals to the testing paradigm would have seriously jeopardized the overall quality of the data due to time constraints of the testing paradigm. Thirdly, additional controls were not tested due to the

project budget. Therefore, the data set for the NS and IS groups that was obtained when establishing the effects of morphine was employed to demonstrate the behavioural effects of 1001 and 1003. Similarly, both drugs were given at the end of 2nd LH induction session on day 2 and before the conduction of each depression test on days 4 to 6 (**Figure 56A**). As expected, exposure to IS significantly increased number of escape failures in stressed rats (T1: NS: 9.200 ± 2.853 attempts, IS: 25.875 ± 1.125 attempts; T2: NS: 5.400 ± 1.990 attempts, IS: 24.250 ± 1.601 attempts; T3: NS: 5.400 ± 3.265 attempts, IS: 22.500 ± 1.452 attempts) (repeated 2-way ANOVA, time: $F(2, 47) = 0.03207$, $p=0.9685$; treatment: $F(2, 47) = 44.50$, $p<0.0001$; time × treatment: $F(4, 47) = 0.8136$, $p=0.5229$). However, in contrast to the previous results with morphine (**Figure 42B**), no significant changes to the number of escape failures were observed in 1001 or 1003-treated animals compared to the IS group (**Figure 55B**).

A

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1001/1003		At the end of I2		Before each LH test		
LH	I1	I2	Interphase interval	T1	T2	T3

B

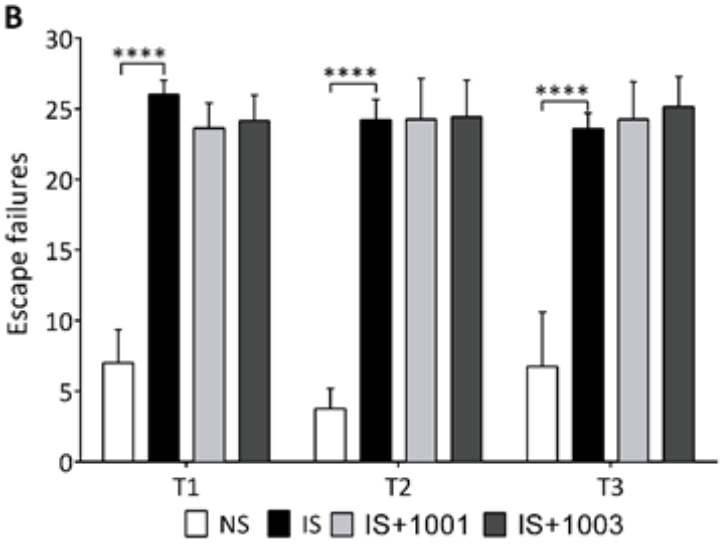

Figure 56. Effects of novel opioids on depressive-like symptoms.
Saline-treated rats received either no stress (NS, n=5) or were exposed to inescapable stress (IS, n=6) on days 1 and 2. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) at the end of day 2 and from days 4 to 6 (A). The data represents the number

of escape failures measured over 3 consecutive testing (T) sessions in the learned helplessness (LH) model (B). $^{****}p<0.0001$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM. The data set for the NS and IS groups was also used for Figure 42B.

5.2.1.2 Effects of 1001 and 1003 on avoidance learning

Based on previous results, morphine enhancing avoidance learning in rats subjected to the LH paradigm. Because 1001 and 1003 display affinity for the MOP receptor similar to morphine, we hypothesized that our bifunctional opioids could also influence learning and memory of the treated animals. It is important to stress that cognition is expected to significantly affect the performance of animals in the LH model (82). Since exposure to IS can lead to memory loss (683), it was essential to confirm that the increased escape failures observed in 1001 and 1003-treated rats was not a consequence of drug-induced impairment of learning and memory.

Therefore, the number of avoidance events was measured as indicator of avoidance learning. In the 1st test, a trend towards reduced avoidance events was detected in the stressed animals and novel opioids-treated rats (**Figure 57**). In the 2nd test, the novel opioids (IS: 3.125 ± 1.008 attempts; 1001: 0.625 ± 0.263 attempts, $p=0.0445$; 1003: 0.571 ± 0.202 attempts, $p=0.0485$) significantly reduced the number of avoidance events compared to IS group (repeated 2-way ANOVA, time: $F(2, 72) = 4.089$, $p=0.0208$; treatment: $F(3, 72) = 8.156$, $p<0.0001$; time × treatment: $F(6, 72) = 0.9319$, $p=0.4776$) (**Figure 57**). The same pattern was observed in the 3rd test, but the differences did not reach statistical significance. Overall, no significant changes on the number of avoidance events were seen between IS and NS groups (**Figure 57**).

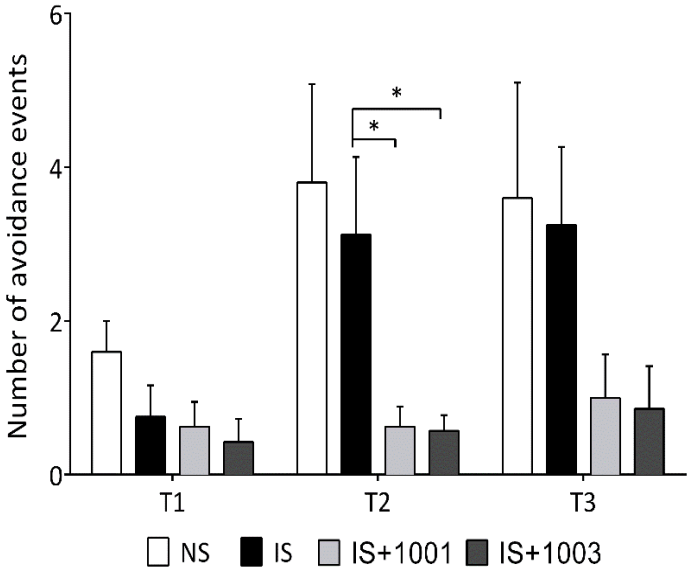

Figure 57. Effects of novel opioids on avoidance learning.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress training (IS, n=6) on days 1 and 2. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6 (for timeline see Figure 55A). The data represents the number of escape failures that were measured over 3 consecutive testing (T) sessions on days 4 to 6. $^{*}p<0.05$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM. The data set for the NS and IS groups was also used for Figure 43.

5.2.1.3 Effects of 1001 and 1003 on escape learning

Other than avoidance learning, escape learning is also one important factor in the LH model. This type of learning indicates how quickly the animals respond to the shocks and how well they remember the escape pattern in the testing trials. To investigate the effects of 1001 and 1003 on escape learning, mean escape latency was measured. In the 1st test, a significantly decreased mean escape latency in the IS group (2.520 ± 0.557 attempts) was observed compared to the NS group (4.032 ± 0.109 attempts) (repeated 2-way ANOVA, time: $F(2, 54) = 1.336$, $p=0.2716$; treatment: $F(2, 54) = 11.31$, $p<0.0001$; time × treatment: $F(4, 54) = 1.219$, $p=0.3135$). While in the 2nd and 3rd tests, no significant changes were observed in all groups. (**Figure 58**).

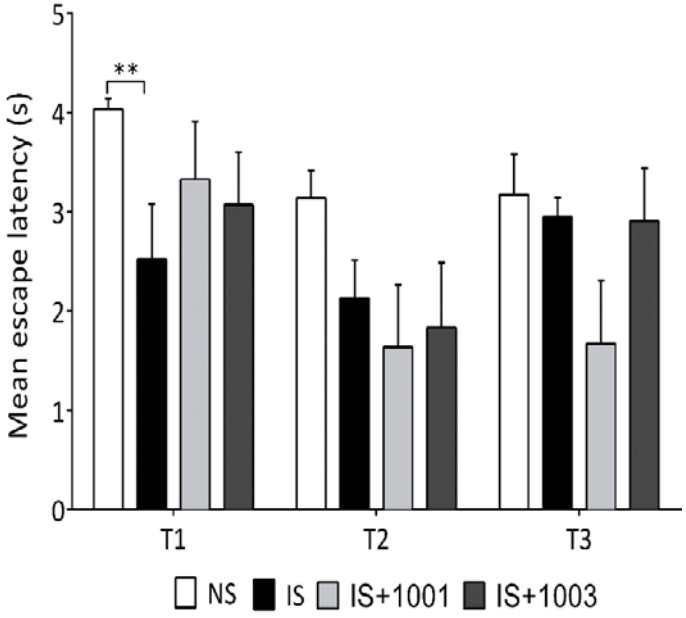

Figure 58. Effects of *n*ovel opioids on escape learning.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6 (for timeline see Figure 55A). The data represents the mean escape latency measured over 3 consecutive testing (T) sessions from days 4 to 6. $^{**}p<0.01$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM. The data set for the NS and IS groups was also used in Figure 44.

5.2.1.4 Effects of 1001 and 1003 on physical activity

In the LH model, transitions between two compartments of the LH chamber during intertrial intervals (i.e., intertrial interval transfers, ITTs) can be used as an index of physical activity, which is known to affect the results of the LH model. It was conceivable that exposure to IS might reduce physical activity of 1001- and 1003-treated rats. Therefore, those animals could present with more escape failures in the LH paradigm. In order to evaluate the influence of the novel bifunctional opioids on the physical activity of the treated rats, the number of ITTs was measured. Between the NS and IS groups, no significant changes of ITTs were detected over three testing sessions. In contrast to morphine (**Figure 45**), 1001-treated animals exhibited a significant decrease in the number of ITTs in the 3rd test (IS: 7.125 ± 0.915 attempts, 1001: 2.625 ± 1.068 attempts, p=0.0317) (repeated 2-way ANOVA, time: F (2, 48) = 9.071, p=0.0005; treatment: F (3, 24) = 3.933, p=0.0205; time × treatment: F (6, 48) = 0.8804, p=0.5165), compared to IS group, but no significance was observed in 1003-treated rats across all testing sessions (**Figure 59**).

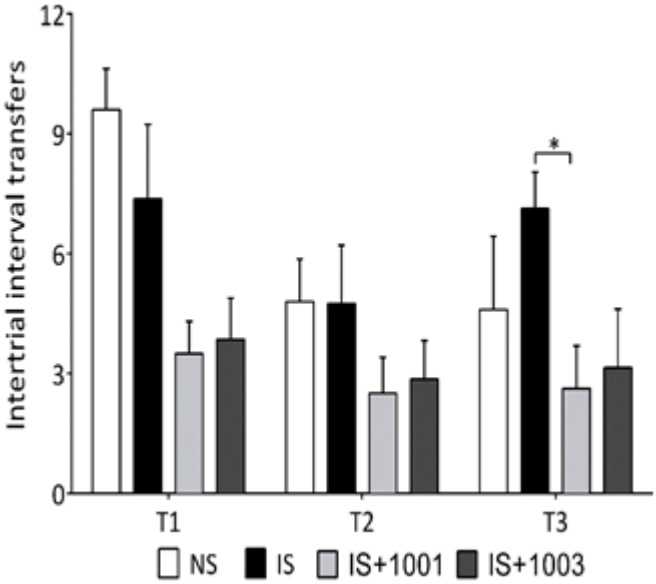

Figure 59. Effects of novel opioids on physical activity.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6 (for detailed timeline see Figure 55A). The data presents the number of intertrial interval transfers (ITT) measured over 3 consecutive testing (T) sessions from days 4-6. $^{*}p<0.05$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM. The data set for the NS and IS groups was also used for Figure 45.

5.2.1.5 Effects of 1001 and 1003 on anxiety-like symptoms

Another confounding factor of the LH model is anxiety, which is known to be potentially induced by exposure to stress (657). Given that 1001 and 1003 did not affect stress-induced escape failures (**Figure 56**) compared to morphine treatment (**Figure 42B**) we investigated if this effect could be due to increased drug-induced anxiety-like symptoms. We therefore measured the freezing time during the intertrial intervals as an index of anxiety in our LH model. Overall, stressed-rats showed increased freezing times compared to the non-stressed rats. A significant increase in freezing time was observed in the IS group (262.225 ± 41.548 s) in the 2nd testing session compared to the NS group (82.820 ± 21.387 s) (repeated 2-way ANOVA, time: F (2, 48) = 9.071, p=0.0005; treatment: F (3, 24) = 3.933, p=0.0205; time × treatment: F (6, 48) = 0.8804, p=0.5156). In both the 1001- and 1003-treated groups, no significant drug-dependent changes to freezing times were observed compared to the IS group (**Figure 60**).

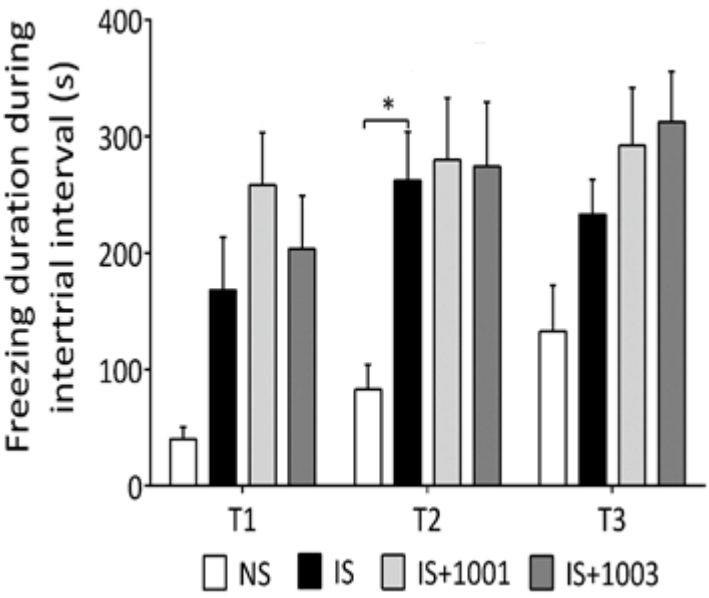

Figure 60. Effects of novel opioids on anxiety-like behaviour.
Saline-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 1 and 2. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6 (for timeline see Figure 55A). The data represents the freezing duration during the intertrial intervals measured over 3 consecutive testing (T) sessions from days 4 to 6. $^{*}p<0.05$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM. The data set for the NS and IS groups was also used for Figure 46.

5.2.2 Psychopharmacological effects of 1001 and 1003 in the novel object recognition model

My previous results demonstrated that morphine-treated rats showed an increased number of avoidance events compared to the stressed saline-treated rats (**Figure 43**), indicating a possible morphine-induced enhancement of learning. Because 1001 and 1003 can activate MOP receptors as morphine does, we assumed that our bifunctional opioids might also improve the learning and memory abilities of our rats. In order to assess the cognitive effects of 1001 and 1003, the NOR model, was used as a widely established learning/memory model. The NOR model is particularly attractive because it requires no external motivation, reward, or punishment and only little habituation. Furthermore, this model can be completed in a relatively short time (684). Briefly, the NOR model contains 3 phases, a habituation phase (the animals familiarize the experimental environment), an induction phase (2 identical objects are used) and a testing phase (one of the familiar objects is replaced with a novel one). In our model, the object learning phase of the NOR model was conducted 24 hours after the 2nd shock induction of the LH model on day 4. The learning phase contained two sessions with an approximate 7 h interval. The short-term and long-term memory of animals were measured 24

and 72 h after the 1st object learning session respectively (**Figure 61A**). In this model, DI was used to display the difference of exploration time between familiar and novel objects.

In both object-learning sessions, all groups of rats spent similar amounts of time exploring both identical objects (**Figure 61B, 60C**). In both the short-term test, non-stressed rats spent significant more time exploring the novel object (novel object: 51.848 ± 12.499 s, familiar object: 12.316 ± 3.678 s, $p<0.0001$) (repeated 2-way ANOVA, time: F (3, 44) = 8.401, $p=0.0002$; treatment: F (1, 44) = 15.39, $p=0.0003$; time × treatment: F (3, 44) = 8.726, $p=0.0001$) (**Figure 61D**). However, no significant changes to exploration time between familiar and novel objects were observed in the IS, 1001- and 1003-treated groups (**Figure 61D**). In the long-term memory test, the non-stressed rats still spent significantly more time with the novel object as expected (novel object: 20.132 ± 6.743 s, familiar object: 5.494 ± 1.381 s) (repeated 2-way ANOVA, repeated 2-way ANOVA, time: F (3, 44) = 8.401, $p=0.0002$; treatment: F (1, 44) = 15.390, $p=0.0003$; time × treatment: F (3, 44) = 8.726, $p=0.0001$) (**Figure 61E**). In the NOR model, the DI was used to quantify how performance of the rats on exploration novel and familiar objects. A negative value of DI indicates memory loss. The DI values in the IS group were significantly lower than that of the NS group in the short-term (IS: -0.154 ± 0.139, NS: 0.568 ± 0.115) (unpaired *t* test, t (10) = 3.762, $p=0.0100$) (**Figure 61F**) and long-term (IS: -0.186 ± 0.112, NS: 0.526 ± 0.142) (unpaired *t* test, t (10) = 3.989, $p=0.0026$) (**Figure 61G**) memory tests. No significant changes on the DI values were observed between 1001-, 1003-treated rats and stressed rats in both memory tests.

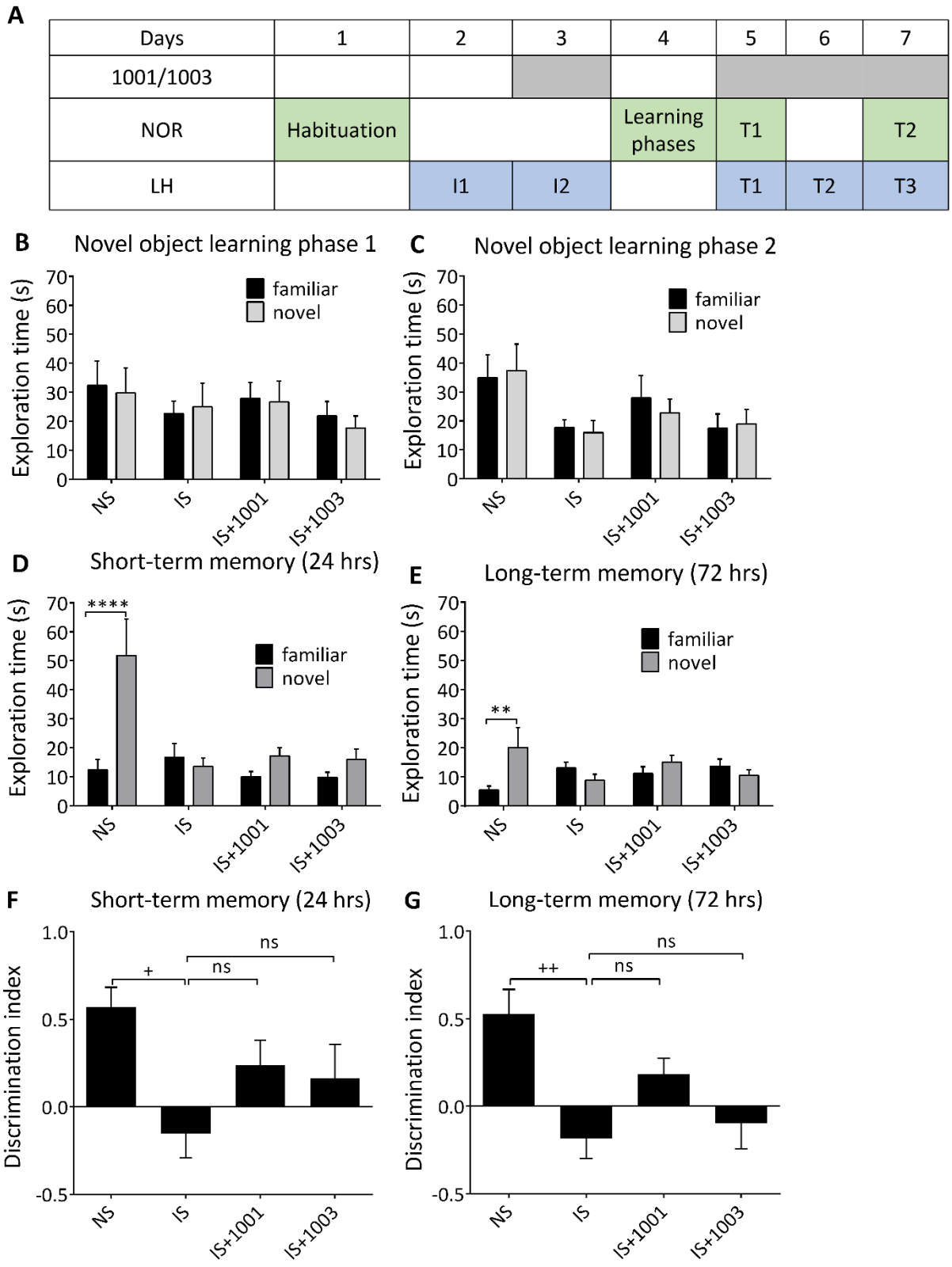

Figure 61. Effects of bifunctional opioids on recognition memory.
Vehicle-treated Sprague Dawley rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6. The novel object recognition (NOR) model was conducted in parallel to the LH procedure (A). Two object learning sessions were

conducted on day 4 using 2 identical objects, which were placed in either the left (L) or right (R) position of the arena. There was 7-hour interval between two sessions (B, C). Basal preference to selected objects were assessed in 15-min learning sessions. Twenty four hours after the first learning session, the short-term memory of rats was tested in a 5 min retention test on day 5 and one of the familiar objects was replaced with a novel object (D). On day 7 the long-term memory was assessed 72 h after the first learning session using a different novel object over 5 min period (E). $^{**}p<0.01$ and $^{****}p<0.0001$ using two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM, ns=no significance. The DI was calculated to illustrate the difference of exploration time between familiar and novel objects in the different groups in the short-term (F) and long-term (G) memory tests. $^{+}P<0.01$ and $^{++}p<0.01$ using two-sided unpaired *t* test.

5.2.3 Psychopharmacological effects of 1001 and 1003 in the open field model

5.2.3.1 Effects of 1001 and 1003 on physical activity

Our results showed overall decreased numbers of intertrial interval transfers in both bifunctional opioids-treated groups (**Figure 59**), which indicates decreased physical activity. Therefore, the OF model as a model of locomotion was used to confirm these effects. The locomotion tests of the OF model were carried out in parallel to the LH procedure. The physical activities of rats were represented as moving duration over a 5-min OF testing period. The physical activity of 1001- and 1003-treated rats were assessed daily from days 2 to 7 (**Figure 62A**). Overall, however, treatment with 1001 or 1003 did not affect moving duration in the stressed animals. (**Figure 62B**).

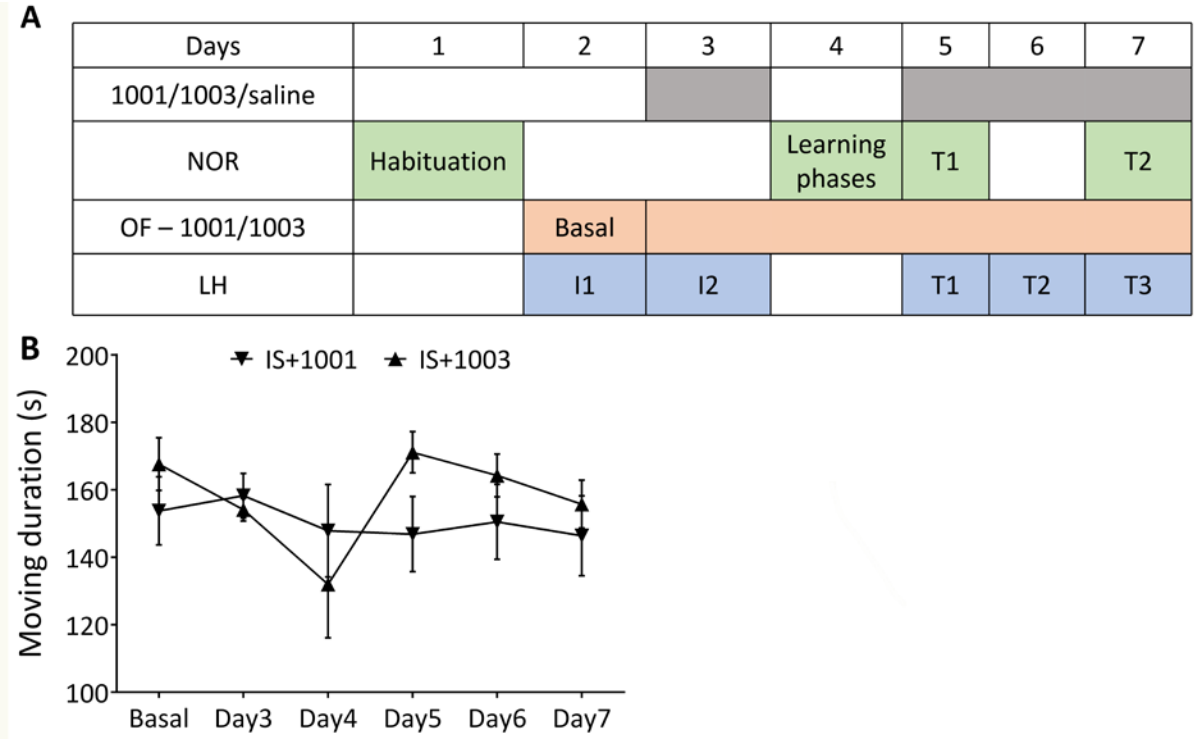

Figure 62. Effects of novel bifunctional opioids on physical activity.
UTA1001 and 1003-treated rats received inescapable stress training on days 2 and 3. Animals were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6. The open field tests were performed prior to the conduction of the LH procedure (A). The physical activity of 1001 and 1003 groups was measured from days 3-7 (B).The data represents time spent moving in the OF arena over a 5-min observation period. Data was analysed using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests.

5.2.3.2 Effects of 1001 and 1003 on anxiety-like symptoms

Percentage of time spent in the central area of the OF chamber can be used as an index of anxiety-like behaviour. No significant changes to the percentage of time spent in the central area of the OF chamber were observed between groups over six testing sessions (**Figure 63**). However, a significant but transient decrease of % time spent in the central area was found for day 4 in the 1003-treated group (6.125 ± 1.080 %), compared to its basal level (20.740 ± 7.414 %) (repeated 2-way ANOVA, time: F (5, 70) = 3.265, p=0.0105; treatment: F (1, 14) = 7.222, p=0.0177; time × treatment: F (5, 70) = 1.010, p=0.4185) (**Figure 63**).

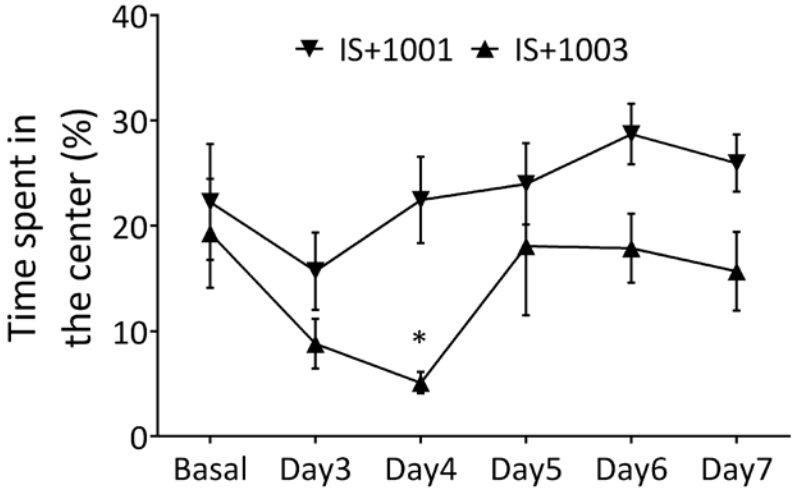

Figure 63. Effects of novel opioids on anxiety-like symptoms.
The anxiety-like behaviour of rats was measured using the open field model and was represented as the percentage of time spent in staying at the central area of the OF arena in a 5 min test (for timeline see Figure 61A). The anxiety-like behaviours of 1001 and 1003-treated groups were measured from days 2-7 (B). $^{*}p<0.05$ compared between basal and day 4 in the 1003-treated group using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; Error bar=SEM.

5.2.4 Effects of 1001 and 1003 on nociception

Activation of both MOP and DOP receptors can produce analgesic effects (685). Since bifunctional opioids 1001 and 1003 showed agonistic activity towards the MOP receptor, we hypothesized that both drugs could have pain-killing effects. Previously, the analgesic effects of 1001 (also called UFP505) were been demonstrated using the tail flick (TF) test (682). To confirm the analgesic effect of 1001 and 1003 in our model, pain perception of 1001- and 1003-treated rats were measured using the TF test, which was performed daily before the conduction of the LH procedure. However, no significant changes were observed within either the 1001- or 1003-treated groups over all testing sessions, compared to the basal pain perception levels (**Figure 64B**).

A

Days	1	2	3	4	5	6	7
1001/1003/saline							
NOR	Habituation			Learning phases	T1		T2
TF – 1001/1003		Basal					
TF – saline		Basal					
LH		I1	I2		T1	T2	T3

B

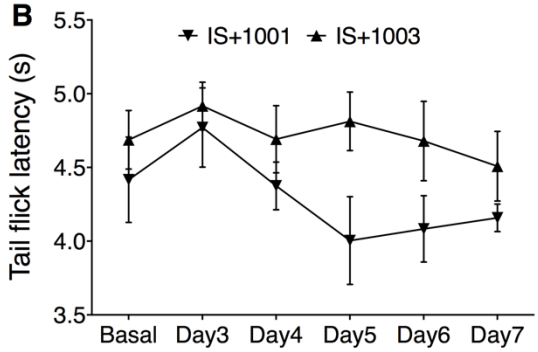

Figure 64. Effects of novel opioids on the pain perception.
Saline-treated rats received inescapable stress (IS, n=6) training on days 2 and 3.Stressed rats were treated with 1001 (5 mg/kg/day, n=8) or 1003 (5 mg/kg/day, n=7) on day 2 and days 4-6. The pain perception of rats was measured in the tail flick (TF) test (A). The TF latency of the 1001- and 1003-treated groups were measured from days 2 to 7 prior to the conduction of the LH procedure (B). The data was analysed using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; Error bar=SEM.

Table 7. Overview of the effects of morphine, imipramine and novel bifunctional opioids in different behavioural models.

(NS: non-stressed, IS: inescapable stress, MOR: morphine, IMI: imipramine; n/a: not measured, EFs: escape failures, ITTs: intertrial interval transfers, ITI: intertrial interval, LD: light and dark, NOR: novel object recognition, TF: tail flick, HP: hot plate, —: no effects, **three arrows: significant changes, one arrow: a trend**)

Groups and parameters	Depression model (LH)					Locomotion model (OF)				Anxiety model (LD)	Learning and memory model (NOR)	Pain models	
	EFs	Avoidance events	Mean escape latency (s)	ITTs	Freezing duration during ITI	Moving duration (s)	%time spent in centre	%travel distance in centre	Rearing duration (s)			TF test	HP test
Measured behaviours	Depressive-like symptom	Avoidance learning	Escape behaviour	Physical activity	Anxiety-like symptom/fear	Physical activity	Anxiety-like symptoms			Anxiety-like symptoms	Recognition memory	Pain perception	
IS	↑↑↑	—	↓↓↓	—	↑↑↑	↓	—	—	—	↓	↓↓↓	↑↑↑	—
IMI	↓↓↓	↑	—	—	—	↓↓↓	—	—	—	n/a	n/a	n/a	
MOR	↓↓↓	↑↑↑	—	↑↑↑	↓↓↓	↑↑↑	—	—	—	↓↓↓	n/a	n/a	
1001	—	↓↓↓	—	↓↓↓	—	—	—	n/a	n/a	n/a	↑	—	n/a
1003	—	↓↓↓	—	—	—	—	—	n/a	n/a	n/a	↑	—	n/a

Chapter 6 Preliminary Investigation of The Relationship Between Monoamine Oxidase, Oxygen Reactive Species and Depression

6.1 *In vitro* investigation of the relationship between MAO and ROS production

MAO produces cytotoxic ligand hydrogen peroxide (H_2O_2) during the metabolism processes of catecholamines. Hydrogen peroxide as a form of oxidative stress can further lead to mitochondrial dysfunction (686). In order to evaluate the relationship between MAO and mitochondria, we measured the amount of MAO-produced ROS using the nonselective MAO substrate tyramine. Since there were no clearly defined protocols to measure MAO-induced ROS, at a first stage, we selected a suitable oxidative stress-responsive probe, optimized exposure time to drugs and established the optimal concentration of tyramine.

6.1.1 ROS production and monoamine oxidase enzyme

The choice of oxidative stress probes

A general ROS indicator (CM-H_2DCFDA) and a lipid peroxidation sensor (BODIPY-C_{11} 581/591) were used to detect MAO-produced ROS. No lipid peroxidation was detected by BODIPY 581/591, whereas significantly increased amount of ROS were detected by the CM-H_2DCFDA from 15-min onwards (0 min: 100.000 ± 7.076 %; 15 min: 155.751 ± 4.139 %; 30 min: 189.807 ± 4.823 %; 45 min: 215.987 ± 8.638 %; 60 min: 232.307 ± 11.246 %; 75 min: 252.945 ± 13.856 %) (2-way ANOVA, time: $F(5, 36) = 35.27$, treatment: $F(1, 36) = 600.1$, time × treatment: $F(5, 36) = 36.98$, $p<0.0001$) (**Figure 65**).

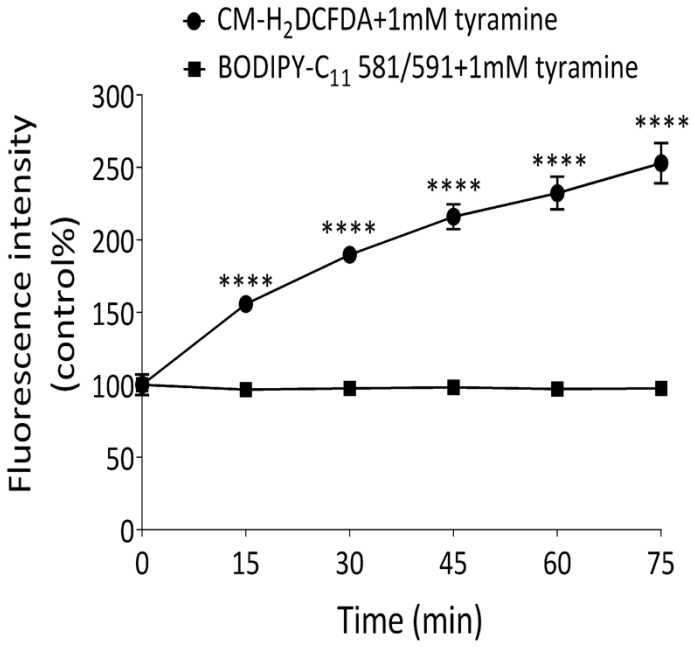

Figure 65. Suitability of different dyes for the detection of MAO-induced reactive oxygen species. Hepatocarcinoma (HepG2) cells were incubated with 1 mM tyramine for up to 75 min and the brief amount of monoamine oxidase (MAO)-produced reactive oxygen species was detected using either CM-H_2DCFDA or BODIPY-C_{11} 581/591. Data represents the percentage of fluorescence intensity relative to the 0 min time point. Statistical significance was determined against the 0 min time point using two-way analysis of variance (ANOVA) followed by Tukey comparison tests; $^{****}p<0.0001$; Error bar=SD; n=6 for each time point.

Optimal exposure time to tyramine

To determine the optimal incubation time, HepG2 cells were exposed to 1 mM tyramine for different time periods up to 75 min and CM-H_2DCFDA fluorescence was detected every 15 min. Overall, tyramine-treated cells produced more ROS compared to the untreated cells. Significant increases in ROS production were observed from 30 min onwards (30 min: control: 133.309 ± 36.366 %, tyramine: 198.787 ± 12.980 %, p=0.0176; 45 min: control: 181.547 ± 65.491 %, tyramine: 262.790 ± 24.978 %, p=0.001; 60 min: control: 227.777 ± 72.547 %, tyramine: 304.929 ± 29.425 %; 75 min: control: 244.333 ± 76.998 %, tyramine: 330.976 ± 33.942 %, p<0.0001). (2-way ANOVA, time: F (5, 27) = 23.16, p<0.0001; treatment: F (1, 27) = 20.75, p=0.0001; time × treatment: F (5, 27) = 0.7546, p=0.5901). However, the changes to ROS levels seen over time in the untreated cells did not reach significance (**Figure 66**).

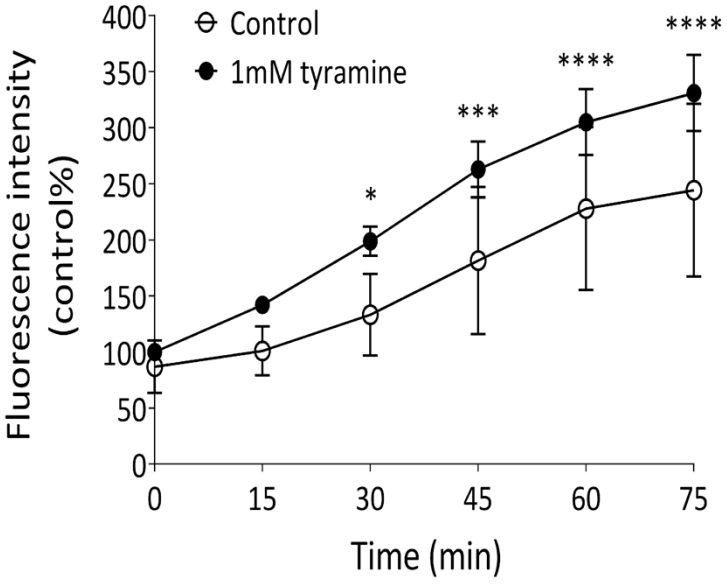

Figure 66. Time-dependent ROS production by tyramine.
HepG2 cells were exposed to 1mM tyramine for up to 75 min. Time-dependent ROS production is displayed as percentage of CM-H_2DCFDA fluorescence intensity relative to the 0 min time point. Statistical significance was determined against the 0 min time point using two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; $^{*}p<0.05$, $^{***}p<0.001$ and $^{****}p<0.0001$; Error bar=SD, n=6 for each time point.

Optimal working concentration of tyramine

The optimal working concentration of tyramine was determined after a 60 min incubation period with HepG2 cells using CM-H_2DCFDA. A range of concentrations of tyramine (0.1 – 1000 µM) were tested in this experiment. Significantly increased amounts of MAO-dependent ROS were observed in a concentration-dependent manner from 1 µM onwards with a maximum at 1000 µM (1 µM: 157.065 ± 12.782 %, p=0.0132; 10 µM: 178.323 ± 17.694 %, p=0.0005; 100 µM: 190.301 ± 13.096 %, p=0.0003; 1000 µM: 191.524 ± 18.482 %, p=0.0003) (1-way ANOVA, treatment: F (5, 12) = 12.53, p=0.0002) (**Figure 67**).

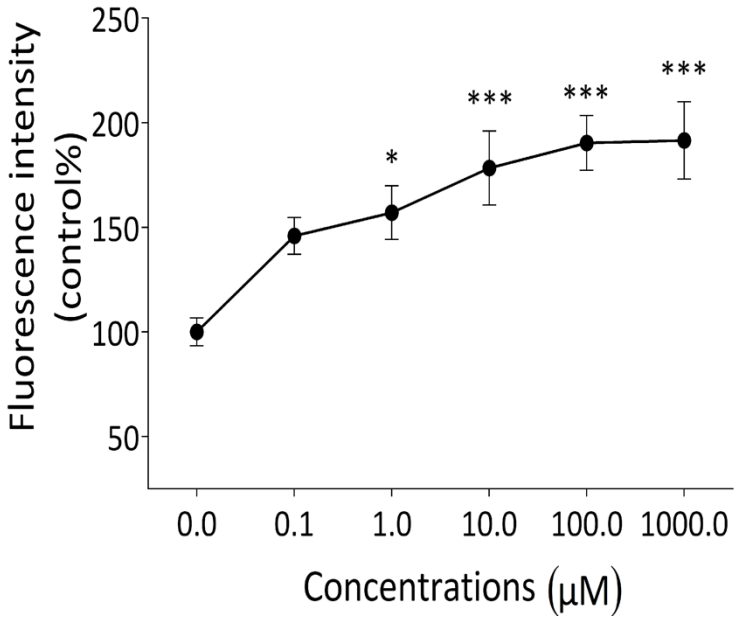

Figure 67. Concentration-dependent ROS production by tyramine.
HepG2 cells were incubated with different concentrations of tyramine (0.1 – 1000 µM) over 60 min incubation period. ROS production is displayed as percentage of fluorescence intensity relative to untreated cells. Statistical significance was determined against the untreated control using one-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons tests with *p<0.05 and ***p<0.001; Error bar=SD.

6.2 Western blot

MAOA is one of the major targets of the conventional antidepressants and this enzyme plays vital role in metabolizing monoaminergic neurotransmitters (687). Increased activity and expression levels of MAOA have been reported in patients with depression (74). Therefore, it was of interest to evaluate potential changes to MAOA expression in rats after exposing them to IS and drugs. For this purpose, western blot (WB) analysis was employed to quantify the expression levels of MAOA in brain homogenates. The homogenates were generated from snap frozen and fresh rat brains. Both hemispheres were used to detect possible differences of MAOA expression in different brain areas. In the present study, an anti-MAOA antibody detected a single band of 68 kDa in rat liver microsomes that were used as positive control (**Figure 68A**), while multiple bands between about 53 to 175 kDa were detected in all brain homogenates (**Figure 68B**), suggesting nonspecific binding of the antibody to brain homogenates. A major band was consistently detected in brain homogenates at around 110 kDa

that did however not correlate to any of the described or expected molecular weights for the MAOA enzyme (**Figure 68B**).

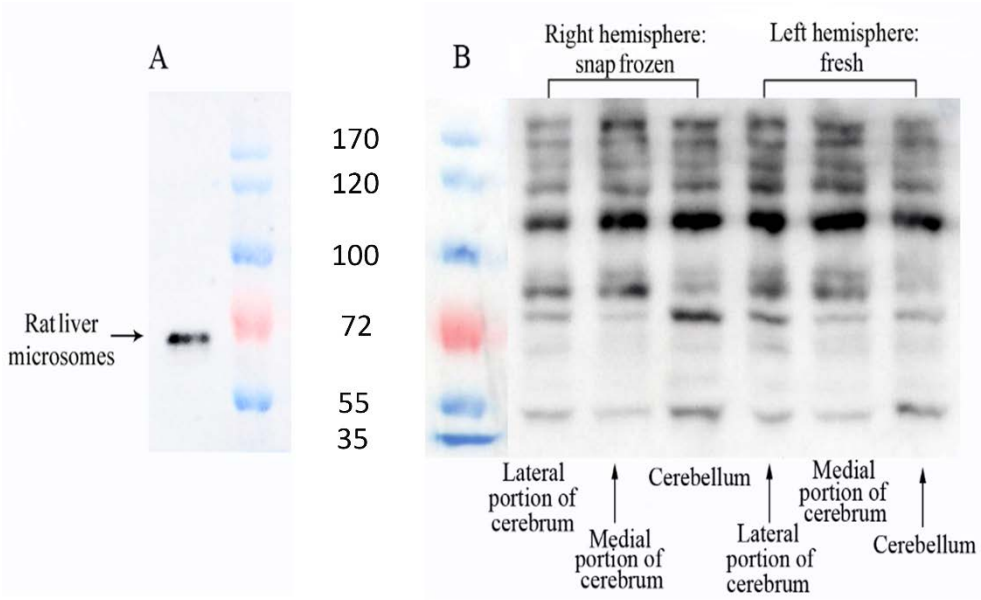

Figure 68. Evaluation of anti-MAOA antibody western blotting.
An anti-MAOA antibody was tested for its ability to detect monoamine oxidase A in rat brain homogenates. Rat liver microsomes (15 µg/lane) were used as a positive control (A). Rat brain homogenates (50 µg/lane) were generated from different regions of both hemispheres (B).

6.3 Histology

6.3.1 MAOA expression

Based on the western blotting results, the selected anti-MAO antibody appeared to be not suitable for quantifying MAOA expression in total brain homogenates. According to the Allen Brain Atlas (http://mouse.brain-map.org/experiment/show?id=74750015), the expression of MAOA mRNA is only observed in very few specialized neuronal cells in the adult mouse brain and it can be expected that this also applies to the situation in rat brain. The present study used total brain extracts since the regions containing MAOA-expressing neurons cannot be dissected from mouse brain. Thus, it was very likely that the quantity of MAOA protein in total brain extract was simply too low to be detectable by western blotting. Therefore, histological analysis was used to investigate the effects of IS and drug treatment on MAO expression using the same

antibody, aiming to specifically stain the MAOA-expressing neurons in brain sections. Since the HPC plays a central role in regulating mood and cognitive function (644), this brain area was selected for histological analysis. MAOA staining appeared as punctuate staining in trails as would be expected for the axonal localisation of a mitochondrial protein (**Figure 69A,B**). In this study, no significant changes in expression levels of MAOA were detected in the HPC of rats after IS exposure, compared to the non-stressed group (**Figure 69C**).

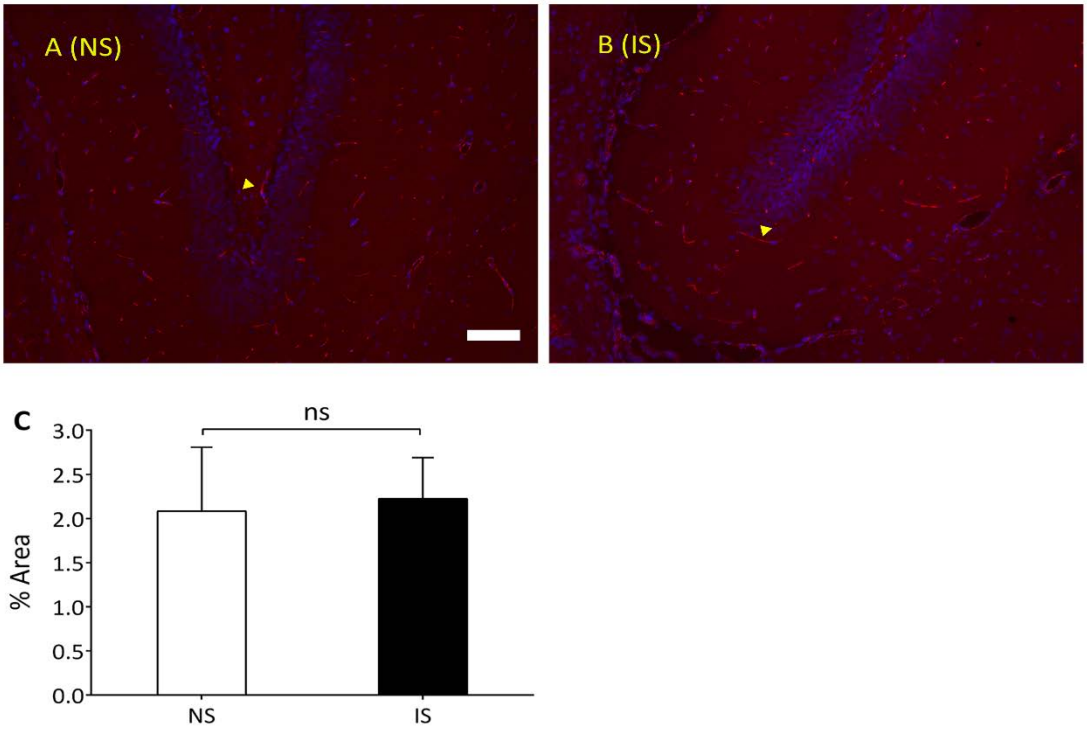

Figure 69. Effect of inescapable foot shocks on MAOA enzyme expression in the hippocampus.
An anti-MAOA antibody was used to detect the expression levels of MAOA in in the hippocampus. Rat brain sections were obtained from the non-stressed (NS, n=5) and the inescapable shock group (IS, n=6). Scale bar indicates 50µM for all images. Images were taken at 20x magnification. Statistics was calculated using Student's *t* test, ns=no significance.

6.3.2 Oxidative stress

Oxidative stress is associated with the development of depressive symptoms (688). Nitrotyrosine, a product of tyrosine nitration mediated by reactive nitrogen species, can be used as an indicator of oxidative cell damage. To evaluate the effects of IS exposure on

inducing oxidative stress damage in anterior and posterior HPC, anti-nitrotyrosine antibody was used. In the anterior HPC, a significant increase in the area of nitrotyrosine staining was observed in the IS group (5.169 ± 1.227 % area), compared to the non-stressed rats (1.248 ± 0.250 % area) (1-way ANOVA, F (3, 19) = 3.715, p=0.0295). Even though decreased nitrotyrosine stain was detected in the 1001- and 1003-treated groups, this change was not significant (**Figure 36**). Significantly increased staining was also detected in the posterior HPC of the stressed rats (5.333 ± 1.659 % area) compared to the NS group (1.287 ± 0.374 % area) (1-way ANOVA, F (3, 16) = 2.730, p=0.0783), while only a trend towards decreased nitrotyrosine staining was observed in the stressed rats that were treated with the bifunctional opioids compared to the stressed animals (**Figure 70**).

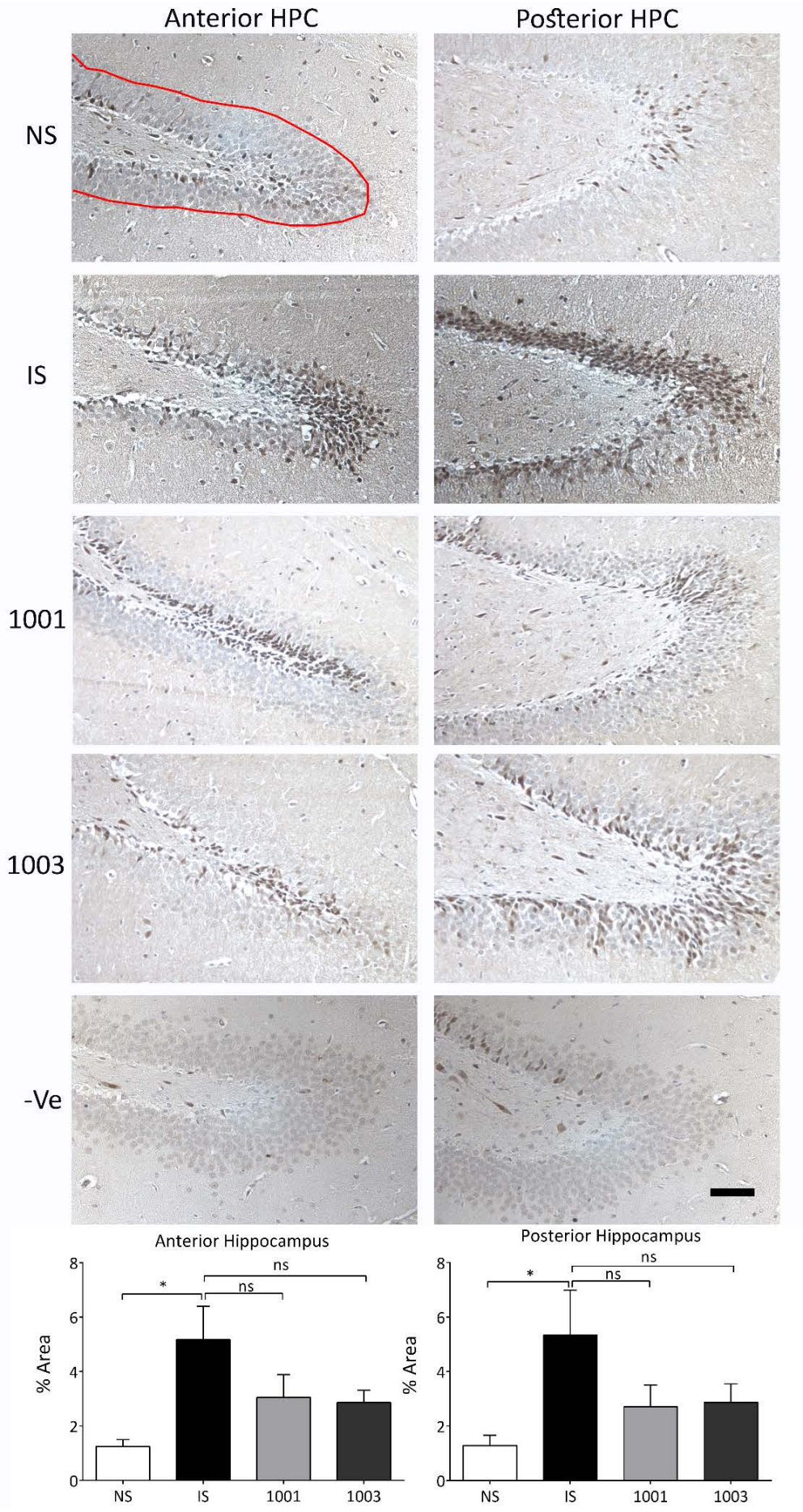
Anterior HPC
Posterior HPC
NS
IS
1001
1003
-Ve
Anterior Hippocampus
Posterior Hippocampus
% Area
0
2
4
6
8
*
ns
ns
NS
IS
1001
1003

Figure 70. Effect of novel bifunctional opioids on stress-induced oxidative cell damage in rat hippocampus.

Oxidative cell damage in the dentate gyrus (DG) areas of the anterior hippocampus (HPC) (left column) and the posterior HPC (right column) was detected using anti-nitrotyrosine antibody. Brain sections were obtained from non-stressed (NS, n=5), inescapable stress (IS, n=5), 1001- (5 mg/kg/day, n=8) and 1003-treated (5 mg/kg/day, n=7) rats respectively. Non-specific rabbit IgG was used as negative control (-Ve). The density of oxidative cell damage was quantified as percentage of stained granule cells (dark brown) in selected DG areas (% area). Scale bar=50µM for 20x magnification. $^{*}p<0.05$ using one-way analysis of variance (ANOVA) followed by Tukey comparison tests; Error bar=SEM, ns=no significance

Chapter 7 The Psychopharmacological Effects of Idebenone

7.1 Histology

The previous results of the 1001- and 1003-treated animals, despite not reaching statistical significance, indicated that stress-induced oxidative damage to the HPC (**Figure 70**) might be related to memory loss (**Figure 61**). Therefore, we hypothesized that prevention of oxidative cell damage might improve depression-related memory loss and possibly also decrease depressive-like symptoms in rats. To test this hypothesis, we used the potent antioxidant idebenone (IDE) in our behavioural models. The antioxidative effect of IDE was confirmed by detecting nitrotyrosine in the HPC of stressed rats. As expected, compared to NS group (anterior: 1.248 ± 0.250 % area; posterior: 1.287 ± 0.374 % area), exposure to IS (anterior: 5.169 ± 1.227 % area; posterior: 5.333 ± 1.659 % area) lead to a significant increase in nitrotyrosine staining in the granule cell layers of hippocampal DG (1-way ANOVA, anterior: $F(3, 18) = 6.944$, $p=0.0027$; posterior: $F(3, 20) = 3.488$, $p=0.0349$) indicative of elevated oxidative stress-induced protein damage. In the anterior HPC, both pre-learning (7-day) and post-learning (3-day) IDE treatment significantly decreased nitrotyrosine staining (% area), compared to the stressed rats (pre-learning IDE: $p=0.0163$; post-learning IDE: $p=0.0022$). However, IDE-induced reduction in nitrotyrosine levels in both treatment groups did not reach statistical significance in the posterior HPC, compared to the stressed rats (**Figure 71**).

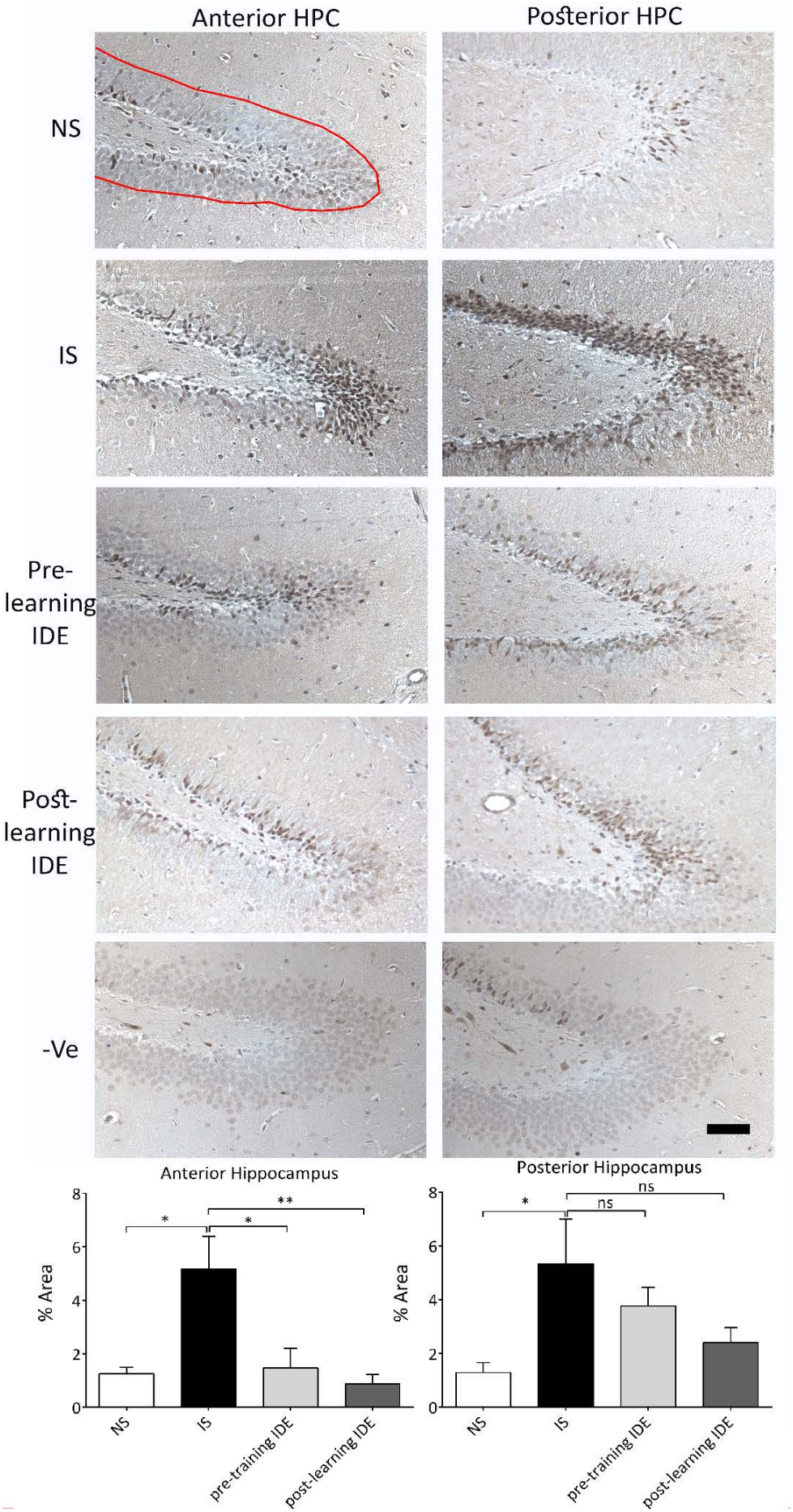
Anterior HPC
Posterior HPC
NS
IS
Pre-learning IDE
Post-learning IDE
-Ve
Anterior Hippocampus
Posterior Hippocampus
% Area
0
2
4
6
8
*
**
ns
NS
IS
pre-training IDE
post-learning IDE

Figure 71. Effects of idebenone (IDE) on stress-induced oxidative cell damage in rat hippocampus.

Oxidative cell damage in the dentate gyrus (DG) area of the anterior hippocampus (HPC) (left column) and the posterior HPC (right column) was detected using anti-nitrotyrosine antibody staining. Brain sections were obtained from non-stressed (NS, n=5), inescapable stress (IS, n=5), 7-day IDE-treated (pre-learning, n=7) and 3-day IDE-treated (post-learning, n=8) rats respectively. Non-specific rabbit IgG was used as negative control (-Ve). The density of oxidative cell damage was quantified as percentage of dark brown granule cells in selected DG areas (% area). Scale bar=50μM. *p<0.05 and **p< 0.01 using one-way Analysis of variance (ANOVA) followed by Tukey comparison tests; Error bar=SEM, ns=no significance. The data set of the NS and IS groups was also used for Figure 34.

7.2 Psychopharmacological effects of idebenone (IDE) in the learned helplessness model

7.2.1 Effect of IDE on depressive-like symptoms

It was proposed that oxidative stress is causally involved in the pathogenesis of depression (408). Therefore not surprising, antioxidants have recently been used as a co-treatment in combination with conventional antidepressants (689). However, this antioxidant approach has led to controversial findings with regards to the treatment of depression (690). We investigated the antidepressant-like effects of IDE, as well as the possible use of antioxidants as antidepressants. Rats that were supplied weekly by the UTAS animal breeding facility were allocated into the control and treatment groups in parallel using a completely randomised method as previously described (634). The antidepressant-like effects of IDE were tested in our LH model in two paradigms, either as a pre-learning treatment (days -3 to 3) or as a post-learning treatment (days 4 to 6) **(Figure 72A)**. As shown previously, exposure to IS significantly increased the number of EFs, compared to the non-stressed rats (T1: NS: 6.000 ± 1.844 attempts, IS: 23.000 ± 2.671 attempts; T2: NS: 3.000 ± 1.0488 attempts, IS: 24.833 ± 1.721 attempts; T3: NS: 8.000 ± 3.507 attempts, IS: 23.667 ± 1.256 attempts) (repeated 2-way ANOVA, time: $F(2, 69) = 0.05162$, $p=0.9497$; treatment: $F(3, 69) = 68.93$, $p<0.0001$; time × treatment: $F(6, 69) = 0.7398$, $p=0.6194$). However, despite potent anti-oxidative activity in the

HPC (**Figure 71**), no significant reduction in escape failures were observed in both IDE treatment groups compared to the IS group (**Figure 72B**).

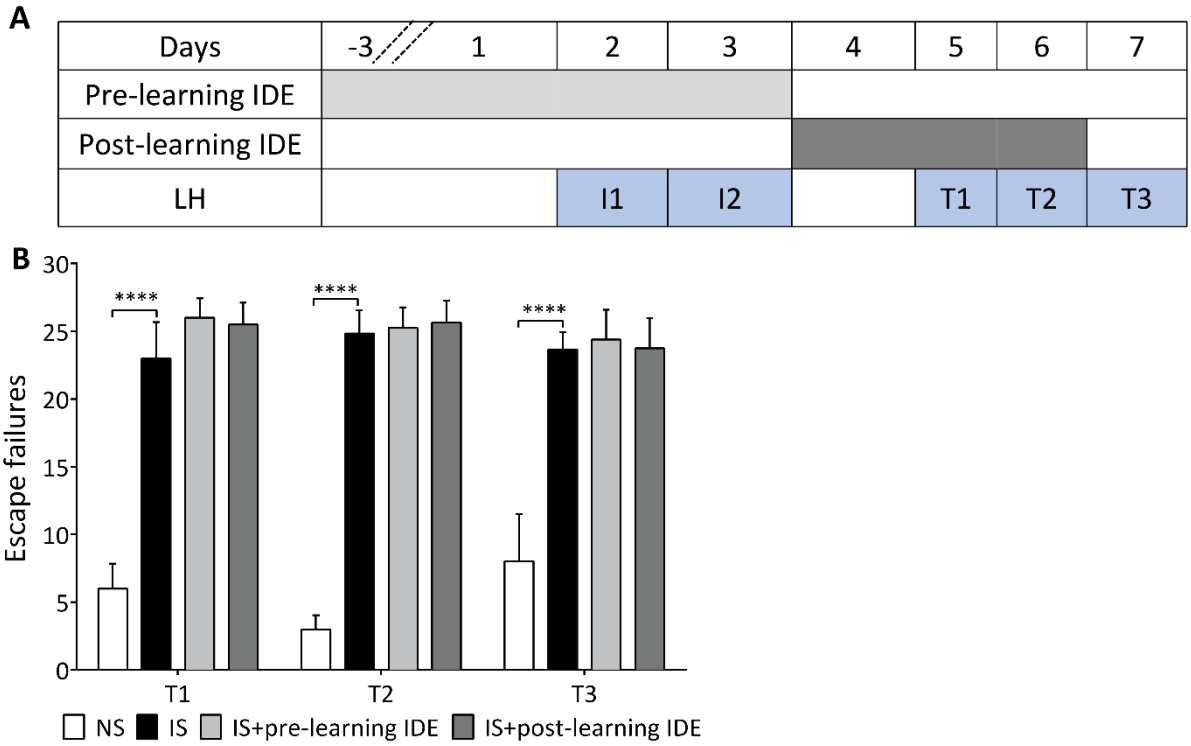

Figure 72. Effects of idebenone (IDE) on depressive-like symptom.
Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8) (A). The data represents the number of escape failures that measured over 3 consecutive testing (T) sessions in the learned helplessness (LH) model (B). ****p<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests, Error bar=SEM.

7.2.2 Effect of IDE on avoidance learning

In the testing phase of the LH model, any changes to the rodents' ability for avoidance learning could conceivably change their escape performance, thus leading to false-positive or false-negative outcomes of drug treatment. IDE has been investigated as a therapeutical approach for AD due to its activity as a cognitive enhancer (691). Therefore, it was important to evaluate the effect of IDE on avoidance learning. In our model, stressed rats (T1: 0.500 ± 0.224 attempts, p=0.0328; T2: 1.333 ± 0.494 attempts, p<0.0001; T3: 0.500 ± 0.342 attempts, p<0.0001) showed significantly less avoidance events compared to the non-stressed rats (T1: 2.600 ±

0.900 attempts, T2: 8.800 ± 2.200 attempts, T3: 10.400 ± 3.900 attempts) over three testing session (repeated 2-way ANOVA, time: F (2, 66) = 4.592, p=0.0136; treatment: F (3, 66) = 27.82, p<0.0001; time × treatment: F (6, 66) = 3.451, p=0.0050). However, no significant changes were observed in both IDE-treated groups compared to the stressed group (**Figure 73**).

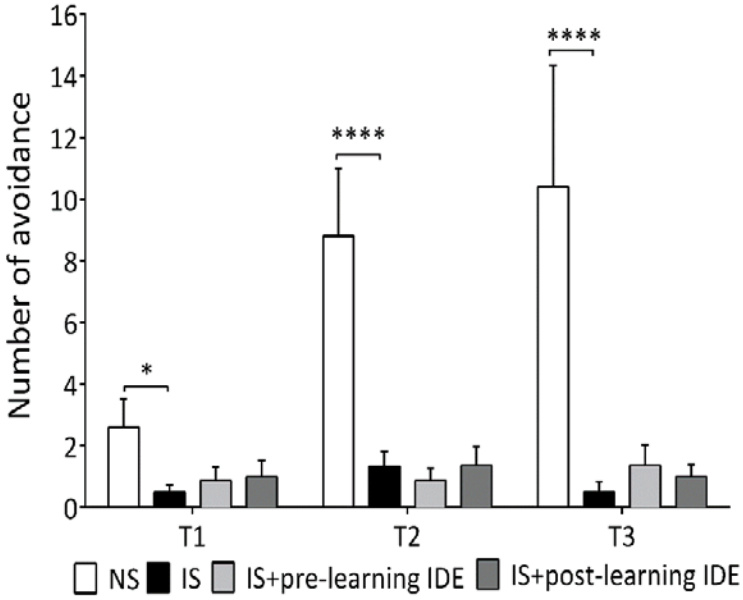

Figure 73. Effects of idebenone (IDE) on avoidance learning.
Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8) (for timeline see Figure 71A). The data represents the number of avoidance events measured over three consecutive testing (T) sessions with 24 h interval between sessions. $^{*}p$<0.05 and $^{****}p$<0.0001 using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests. Error bar=SEM.

7.2.3 Effect of IDE on escape learning

Escape learning is another form of learning that could affect the experimental outcomes of the LH model. Similar to avoidance learning, escape learning can also enhance the escape performance of test animals and could result in reduced escape failures. Therefore, we assessed the effect of IDE on escape learning by measuring the mean escape latency in the LH model, but no significant changes were observed in the treated or untreated groups over three testing sessions (**Figure 74**).

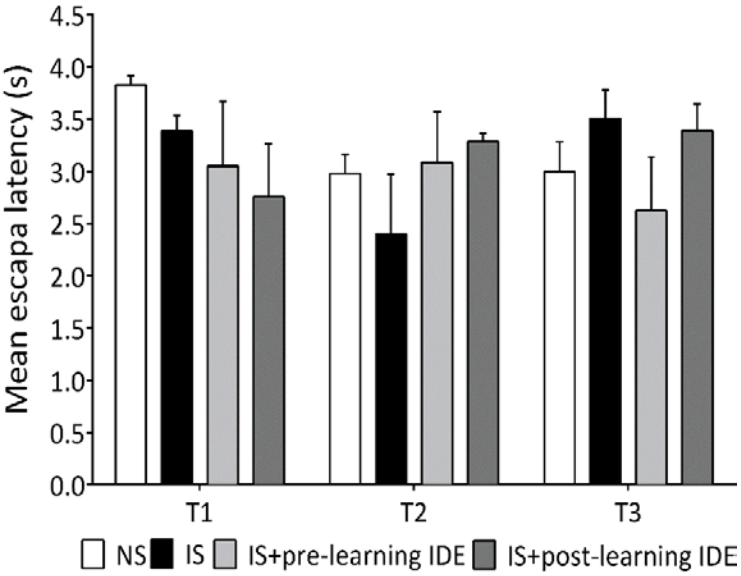

Figure 74. Effects of idebenone (IDE) on escape learning. Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8) (for timeline see Figure 71A). The data represents the average escape latency of each testing trial measured over three consecutive testing (T) sessions with a 24-hour interval between sessions. Statistical analysis was calculated using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

7.2.4 Effect of IDE on physical activity

Less physical activity has been observed in patients with depression (692). Similarly, exposure to stress can also induce reduced physical activity in animals (693). Therefore, it was important to confirm that the observed escape failures in the IDE-treated groups were not a result of reduced locomotion in our rats after exposure to foot shocks in my own study. In the LH model, the number of intertrial interval transfers (ITTs) was measured as an index of physical activity. Overall, no significant changes were observed in any of the groups across all testing sessions (**Figure 75**).

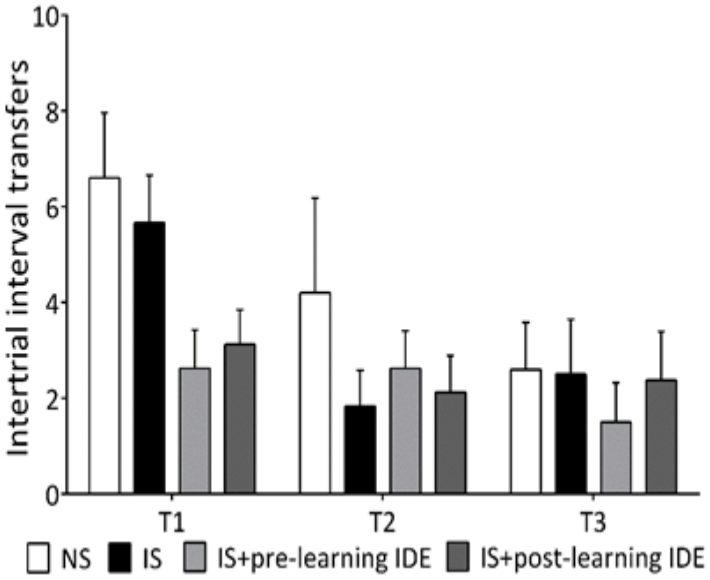

Figure 75. Effect of idebenone (IDE) on locomotion.
Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8) (for timeline see Figure 71A). The data represents the number of intertrial interval transfers measured over three consecutive testing (T) sessions with a 24-hour interval between sessions. Statistical analysis was calculated using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

7.2.5 Effect of IDE on anxiety-like behaviour

Exposure to inescapable foot shocks can induce anxiety-like symptoms (657). To evaluate if the observed escape failures in the IDE-treated groups (**Figure 72**) were a result of anxiety-like symptoms, we measured the freezing time during intertrial intervals in the LH model as an indicator of anxiety. Overall, no significant changes were observed between groups over three testing sessions (**Figure 76**).

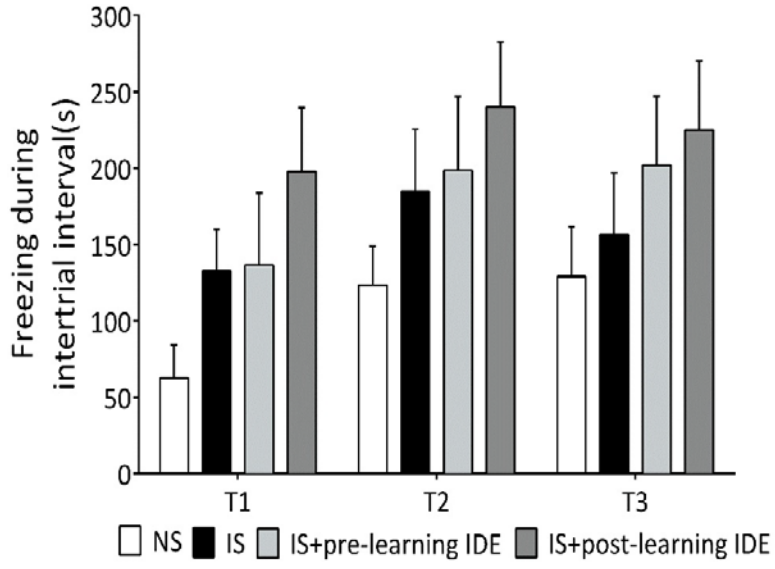

Figure 76. Effects of idebenone (IDE) on anxiety-like behaviour.
Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8). (for timeline see Figure 71A). The data represents the freezing duration during intertrial interval measured over three consecutive testing (T) sessions with a 24 h interval

between sessions. Statistical analysis was calculated using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests. Error bar=SEM.

7.3 Psychopharmacological effects of IDE in the novel object recognition model

IDE has been used to treat cognitive deficits in patients with dementia, demonstrating potent clinical effects by enhancing learning and memory (694). There is also some limited evidence that oxidative stress is involved in the pathogenesis of memory impairment in depression (695). Since IDE is a much more potent antioxidant compared to our bifunctional opioids, we hypothesized that this drug may more effectively reverse depression-associated memory loss via reducing oxidative damage in the brain. Even though no significant changes were observed in the IDE-treated animals with regards to avoidance (**Figure 73**) and escape (**Figure 74**) learning in the LH model, it was still of interest to confirm the effects of IDE in a model of depression-induced cognitive deficits. The memory abilities of rats were accessed in the NOR model. In both object-learning sessions of our model, rats from all groups spent similar amounts of time exploring identical objects (**Figure 77B, 77C**). In the short-term memory test, non-stressed rats spent significantly longer time exploring a novel object than a familiar object (novel object: 51.848 ± 12.499 s, familiar object: 12.316 ± 3.678 s) (repeated 2-way ANOVA, time: $F(3, 46) = 11.00$, $p<0.0001$; treatment: $F(1, 46) = 17.38$, $p=0.0001$; time × treatment: $F(3, 46) = 10.64$, $p<0.0001$). However, no significant changes in exploration time between familiar and novel objects were detected in the IS and IDE-treated groups (**Figure 77D**). In the long-term memory test, significantly less time was spent exploring a familiar object in both the NS and pre-learning IDE-treated groups (novel object: 20.132 ± 6.743 s, familiar object: 5.494 ± 1.381 s) (repeated 2-way ANOVA, repeated 2-way ANOVA, time: $F(3, 46) = 0.7326$, $p=0.5379$; treatment: $F(1, 46) = 4.808$, $p=0.0334$; time × treatment: $F(3, 46) = 6.386$, $p=0.0010$) (**Figure 77E**). In the NOR model, the DI was used to quantify the performance of the rats regarding the exploration of novel and familiar objects. A negative value of DI typically

indicates memory loss, while a positive value indicates memory of the familiar (learned) object. In the short-term memory test, the DI value of the IS group was negative (-0.154 ± 0.139) and was significantly lower than the NS group (0.568 ± 0.115) (unpaired student *t* test, t (10) = 3.989, *p*=0.0037) (**Figure 77F**). In contrast, a significantly higher, positive DI value was detected in the pre-learning IDE-treated (0.405 ± 0.049) group compared to the IS group (unpaired student *t* test, t (12) = 1.270, *p*=0.0015) (**Figure 77F**). In the long-term memory test, stressed rats showed a significantly smaller, negative DI value than the NS group (IS: -0.186 ± 0.112, NS: 0.526 ± 0.142) (unpaired student *t* test, t (10) = 3.989, *p*=0.0026). Similarly, in this test, a significant DI increase was observed in the pre-learning IDE-treated group (0.334 ± 0.146) compared to the IS group (unpaired student *t* test, t (12) = 2.828, *p*=0.0152), while no significant changes were observed in the post-learning IDE-treated groups for both short- and long-term memory (**Figure 77F, 77G**).

A

Days	-3	1	2	3	4	5	6	7
Pre-learning IDE								
Post-learning IDE								
NOR		Habituation			Learning phases	T1		T2
LH			I1	I2		T1	T2	T3

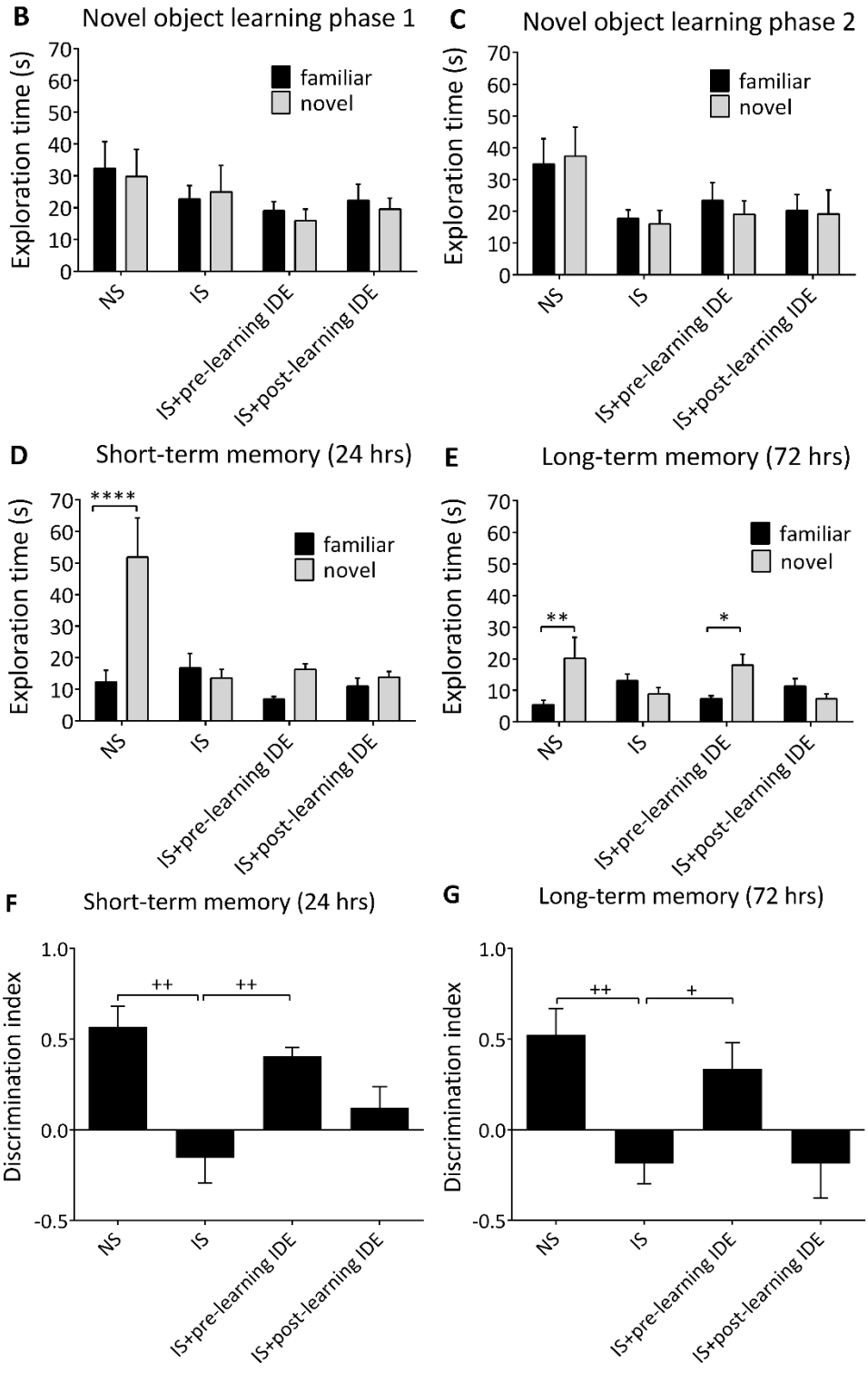

Figure 77. Effects of idebenone (IDE) on recognition memory.
The learning and memory of Sprague Dawley rats was measured in the novel object recognition (NOR) model from days 2 to 7. Vehicle-treated rats received either no stress (NS, n=5) or inescapable stress (IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (pre-learning, n=7) or for 3 days from days 4 to 6 (post-learning, n=8) (A). Two object learning sessions were conducted on day 4 using 2 identical objects, which were placed in either the left (L) or right (R) position of the arena. There was 7-hour interval between these two sessions. Basal preference to selected objects were assessed over 15-min learning period (B,C). The first 5 min memory test was conducted on day 5 using a novel object (D) and the second memory test was conducted on day 7 using a different novel object (E). The DI was used to quantify how performance of the rats on exploration novel and familiar objects through calculating the differences of exploration time on both objects on day 4 (F) and day 7 (G). The data represents the exploration time spent on both objects. $^{*}p<0.05$, $^{**}p<0.01$ and $^{****}p<0.0001$ using repeated two-way analysis of variance (ANOVA) followed by Tukey comparison tests; $^{+}p<0.05$ and $^{++}p<0.01$ using unpaired student *t* test. Error bar=SEM, ns=no significance.

7.4 Psychopharmacological effects of IDE in the open field model

7.4.1 Effect of IDE on physical activity

Our previous results suggested that IDE treatment has no effect on the physical activity of rats (**Figure 74**). To confirm this effect of IDE on locomotion, the OF model was used. No significant differences were observed in the NS (**Figure 78B**), the IS (**Figure 78C**) and the pre-learning IDE-treated (**Figure 78D**) groups over all testing sessions, compared to day 2 within the same groups. The only significant change to moving duration was observed in the post-learning IDE rats on day 4, compared to day 2 (day 4: 138.000 ± 26.970 s, day 2: 192.250 ± 11.467 s, p=0.0002) within the group (repeated 2-way ANOVA, time: F (5, 115) = 4.814, p=0.0005; treatment: F (3, 23) = 1.361, p=0.2796; time × treatment: F (15, 115) = 1.304, p=0.2111) (**Figure 78E**).

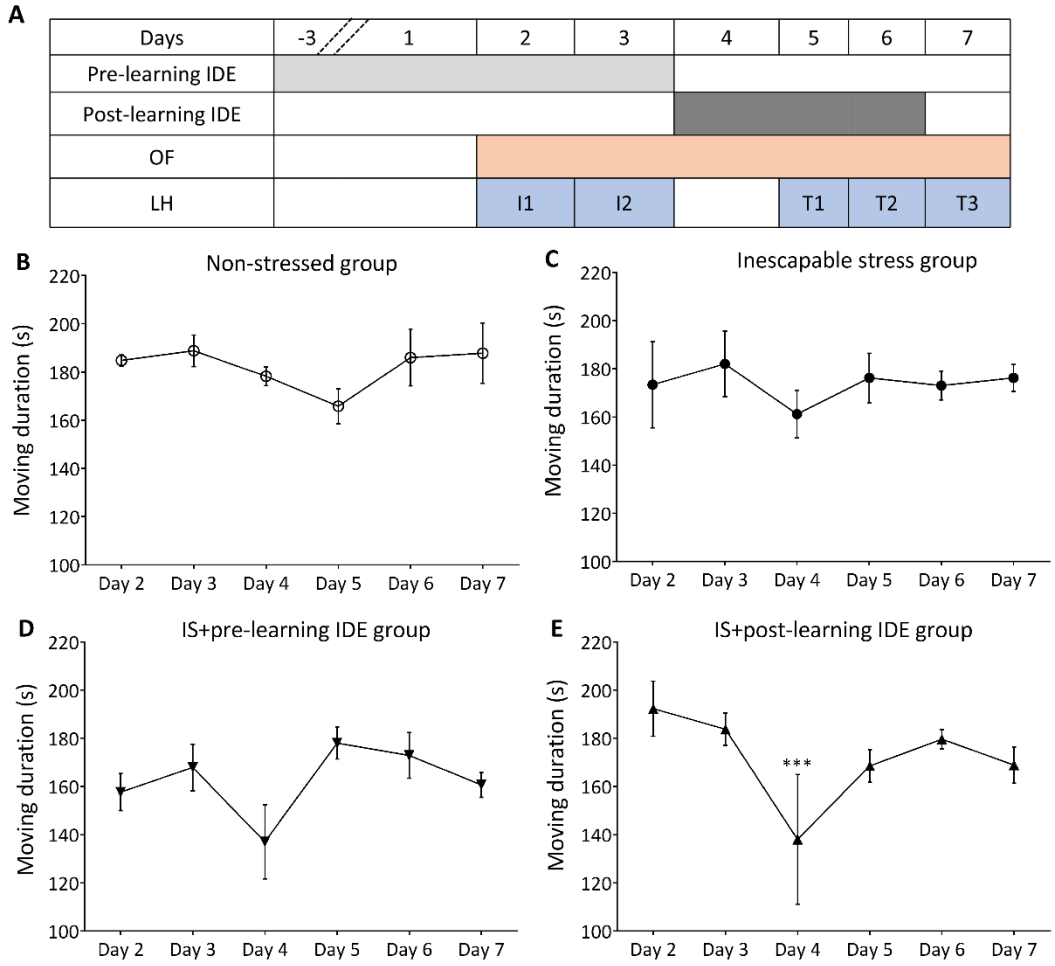

Figure 78. Effects of idebenone (IDE) on physical activity.
The locomotion of rats was measured in the open field test from days 2 to 7 (A). Vehicle-treated rats received either no stress (B, NS, n=5) or inescapable stress (C, IS, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (D, pre-learning, n=7) or for 3 days from days 4 to 6 (E, post-learning, n=8). The time spent moving in the open field area was measured in a 5 min OF test. $^{***}p<0.001$ versus day 2 within the same group using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; Error bar=SEM.

7.4.2 Effect of IDE on anxiety-like symptoms

In the LH model, IDE treatment did not affect freezing time during the intertrial interval (**Figure 75**). To confirm the effects of IDE on anxiety-like symptoms, we measured the percentage of time spent in central area of the OF chamber in the OF model. Similarly, no significant changes were found for all groups, compared to day 2 within each group (**Figure 79**).

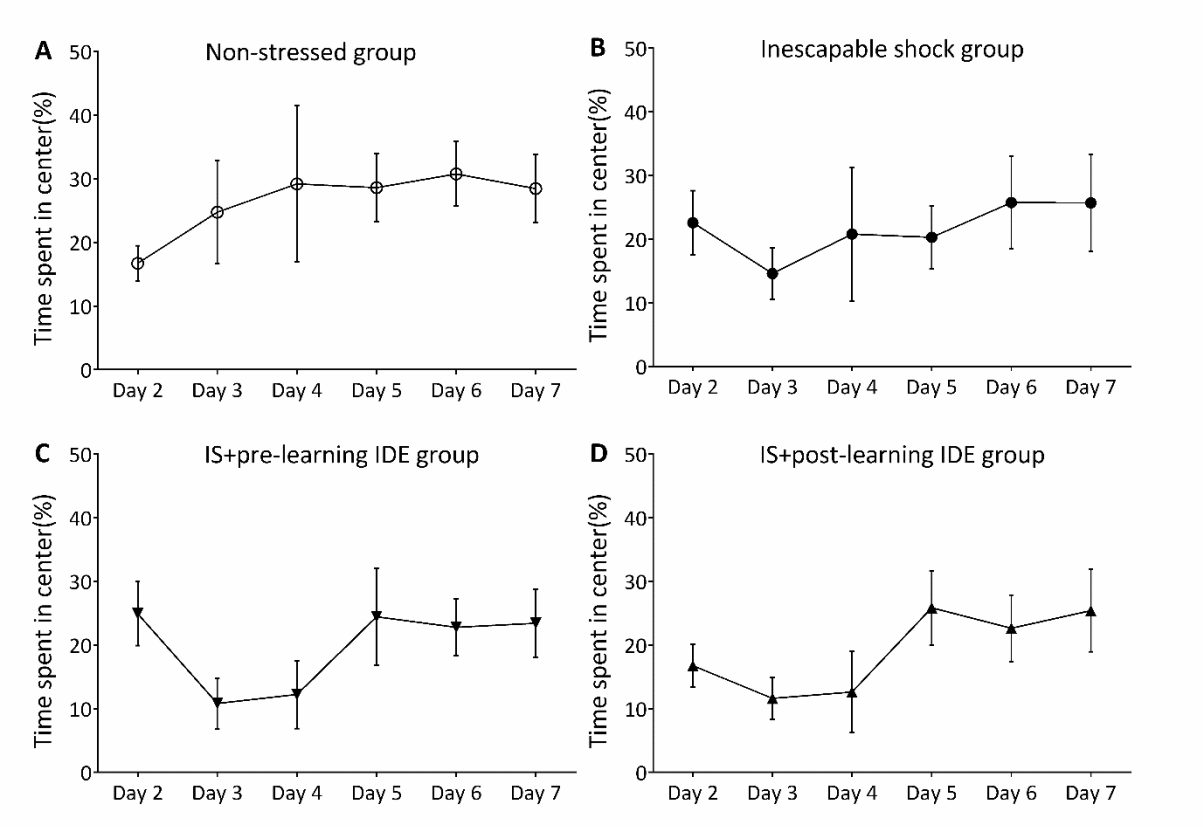

Figure 79. Effects of idebenone (IDE) on anxiety-like symptoms.
Vehicle-treated rats received either no stress (A, NS, n=5) or inescapable stress (B, IS, n=6) training on days 2 and 3 (for timeline see Figure 71A). Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (C, pre-learning, n=7) or for 3 days from days 4 to 6 (D, post-learning, n=8). The percentage of time spent in the central area of the open field area was measured in a 5 min OF test from days 2 to 7. Statistical significance was calculated using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; Error bar=SEM.

7.5 Effect of IDE on nociception

Exposure to IS in the LH model can decrease animals' sensation and increase their fears towards the shocks, thus resulting in more escape failures (669). Based on our previous results, IDE treatment has no effect on depressive-like symptoms in the LH model (**Figure 72B**). To determine if IDE has potential analgesic effects to interfere with its effects in the LH tests, the tail-flick (TF) test was used. Therefore, to test this hypothesis, we investigated the effect of IDE on pain perception in the TF test. However, in our model, no significant changes in pain perception were observed either between or within all groups (**Figure 80**).

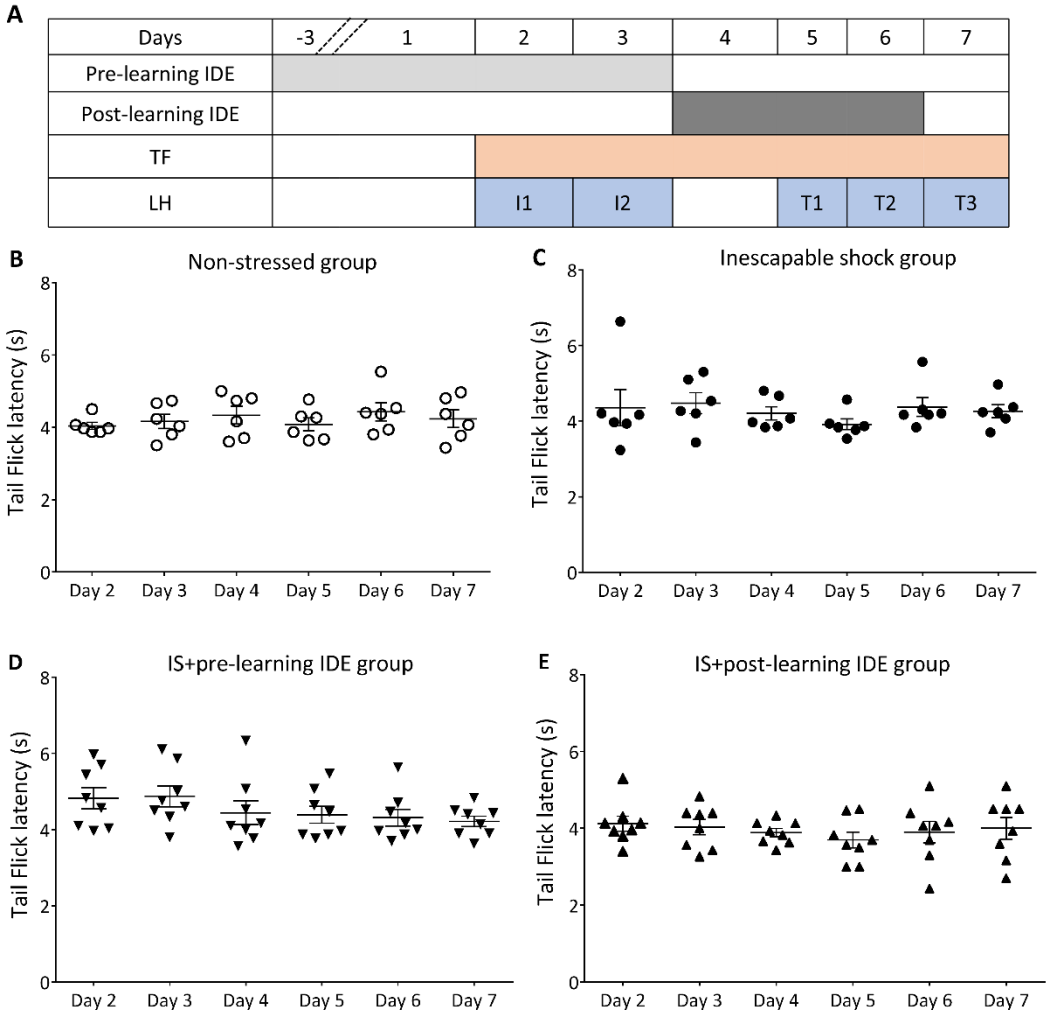

Figure 80. Effects of idebenone on the pain perception.

Pain perception of rats was measured in the tail-flick (TF) test from days 2 to 7 (A). Vehicle-treated rats received either no stress (B, n=5) or inescapable stress (C, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (D, pre-learning, n=7) or for 3 days from days 4 to 6 (E, post-learning, n=8). The tail flick latency was measured 3 times in each test. Statistical significance was calculated using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests; Error bar=SEM.

7.6 Effect of IDE on food consumption and body weight

Obesity can impair cognitive and brain health in humans (696). According to our previous results, IDE treatment was able to reverse stress-induced memory loss in our NOR model (**Figure 77**), suggesting improving effect on cognitive function. However, effect of IDE on body mass is still unknown. In order to assess the effects of IDE on body mass under stressful condition, body weight (BW) and food intake (FI) of all groups were measured daily from days 1 to 7 in our study. FI was standardised on the individual body weight of each rat (=food intake/body weight).

Overall, the BW of all rats increased over the experimental period. In the NS group, the BW showed significant increases from day 3 onwards with a maximum on day 7 (day 3: 299.2 ± 23.483 g, p=0.0004; days 4: 300.4 ± 22.391 g, p<0.0001; day 5: 302.4 ± 21.200 g, p<0.0001; day 6: 308.2 ± 21.754 g, p<0.0001; day 7: 312.0 ± 21.274 g, p<0.0001), compared to day 1 (287.2 ± 23.137 g) within the same group (**Figure 81B**) (repeated 2-way ANOVA, time: F (3, 138) = 18.54, p<0.0001; treatment: F (5, 138) = 1.155, p=0.3347; time × treatment: F (15, 138) = 0.02404, p>0.9999). Similar results for BW were observed in the IS and both IDE-treated groups (**Figure 81C**, **81D, 81E**). However, the FI/BW ratio reduced significantly from days 4 to 7 in the NS and the post-learning IDE-treated groups (**Figure 81B, 81C, 81E**). The lowest food consumption in the pre-learning IDE-treated rats was observed on day 4, which was significant compared to that of day 1 (day 4: 16.000 ± 2.155 g; day 1: 21.875 ± 0.766, p=0.0011) (repeated 2-way ANOVA, time: F (3, 138) = 4.249, p<0.0001; treatment: F (5, 138) = 5.811, p<0.0001; time × treatment: F (15, 138) = 3.553, p<0.0001) (**Figure 81D**).

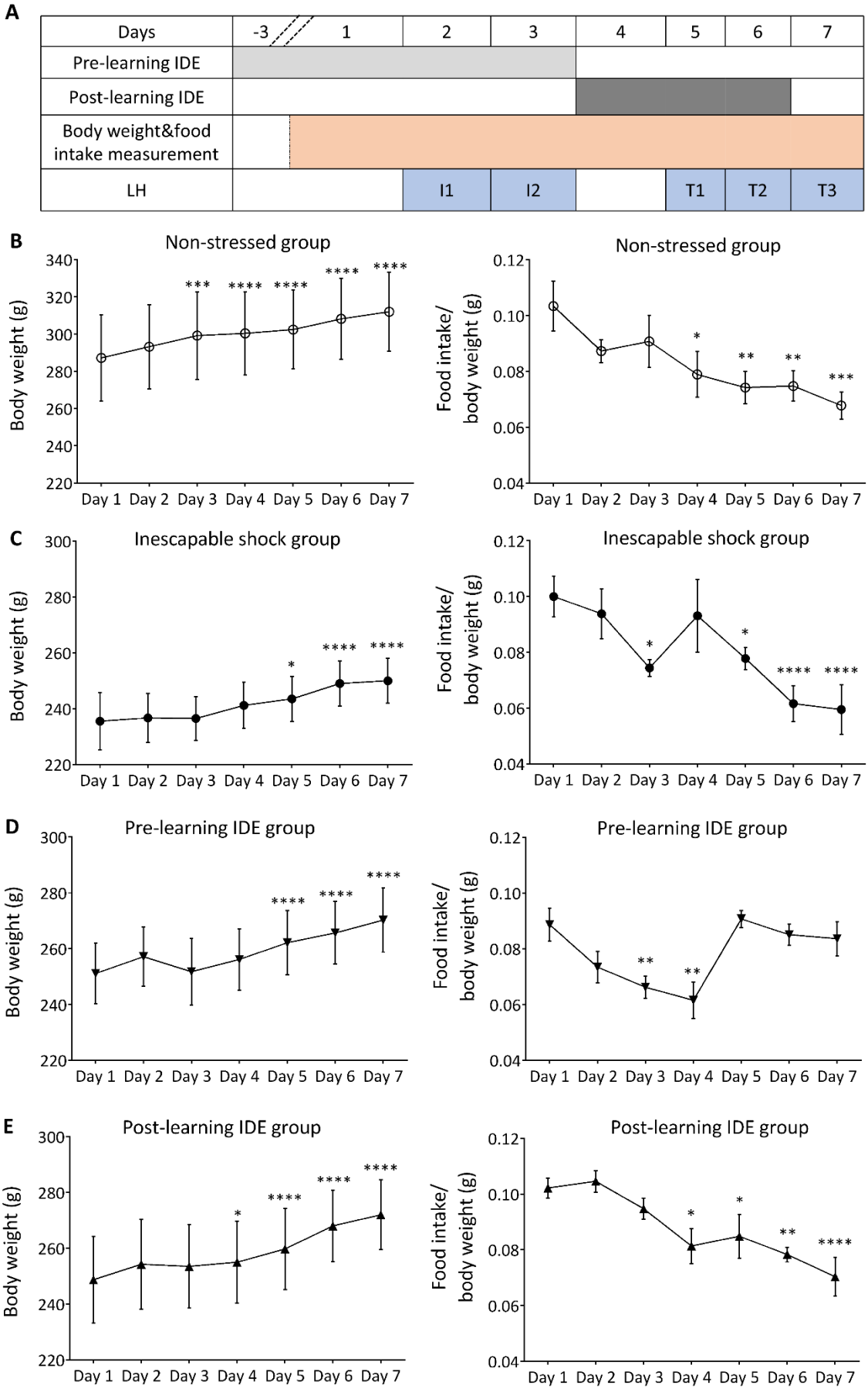
A
Days
-3
1
2
3
4
5
6
7
Pre-learning IDE
Post-learning IDE
Body weight&food intake measurement
LH
I1
I2
T1
T2
T3
B
Non-stressed group
Body weight (g)
Food intake/ body weight (g)
C
Inescapable shock group
D
Pre-learning IDE group
E
Post-learning IDE group
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

Figure 81. Effect of idebenone (IDE) on food consumption under stress.
Body weight and foot intake of rats were measured from days 1 to 7 (A). Vehicle-treated rats received either no stress (B, n=5) or inescapable stress (C, n=6) training on days 2 and 3. Stressed rats were treated with IDE (200 mg/kg/day) for 7 days from days -3 to 3 (D, pre-learning, n=7) or for 3 days from days 4 to 6 (E, post-learning, n=8). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$ versus the food intake amount per body weight on day 1 within the same group using repeated two-way analysis of variance (ANOVA) followed by Dunnett comparison tests, Error bar=SEM.

Chapter 8 Investigation of The Effects of Idebenone On The Fear Extinction

It was interesting to observe that pre-learning IDE-treated rats spent significantly more time exploring the novel object in both short-term and long-term memory tests (**Figure 77**). Therefore, to further investigate the effects of IDE on cognitive function under stressful conditions, we tested this drug in the FE model. The FE model is a frequently used model to study learning and memory functions and serves as a model for PTSD (574). The FE model contains four phases: a habituation, FC, FE and PE phase. Rats received either 7-days IDE treatment or vehicle before the conduction of the FE procedure.

In the FC phase, IDE-treated animals showed more freezing behavior compared to the vehicle-treated group overall. Specifically, significantly more freezing time was observed in the IDE-treated group in trial 3, compared to the vehicle-treated group (vehicle: 1.863 ± 1.863 %, IDE: 31.230 ± 10.050 %, p=0.0168) (2-way ANOVA, trial: F (5, 78) = 9.511, p<0.0001; treatment: F (1, 78) = 13.07, p=0.0005; trial × treatment: F (5, 78) = 1.487, p=0.2037) (**Figure 82C**). In the FE phase, significantly more freezing time was detected at the 1st extinction trial in the IDE-treated rats, compared to untreated rats (vehicle: 12.758 ± 7.665 %, IDE: 20.043 ± 8.007 %, p=0.0473) (2-way ANOVA, trial: F (5, 78) = 9.511, p<0.0001; treatment: F (1, 78) = 13.07, p=0.0005; trial × treatment: F (5, 78) = 1.487, p=0.2037) (**Figure 82D**). In the post-extinction test, no significant differences were observed between the IDE-treated and untreated animals (**Figure 82E**).

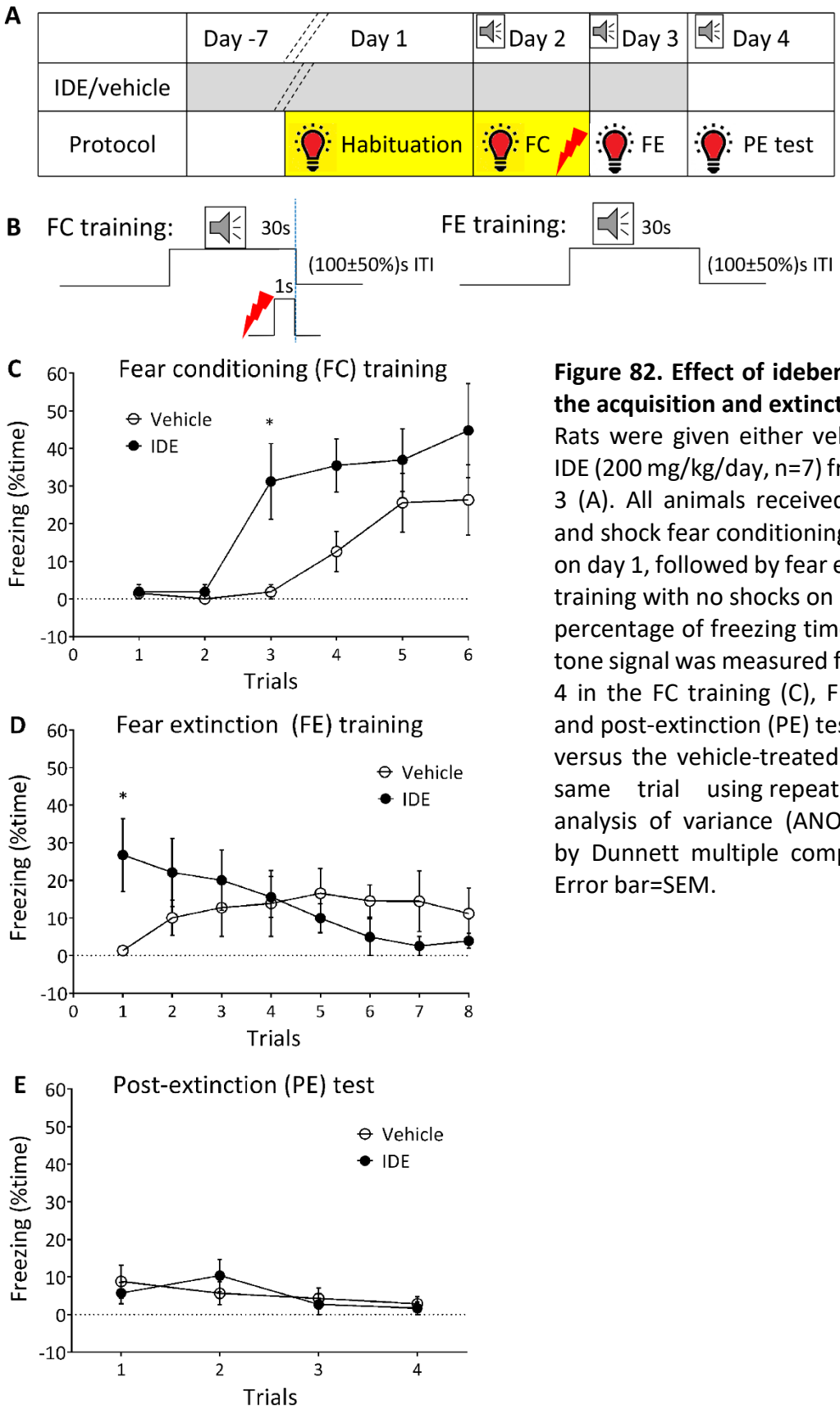

Figure 82. Effect of idebenone (IDE) on the acquisition and extinction of fear. Rats were given either vehicle (n=8) or IDE (200 mg/kg/day, n=7) from days -7 to 3 (A). All animals received paired tone and shock fear conditioning (FC) training on day 1, followed by fear extinction (FE) training with no shocks on day 2 (B). The percentage of freezing time during each tone signal was measured from days 2 to 4 in the FC training (C), FE training (D) and post-extinction (PE) test (E). $^{*}p<0.05$ versus the vehicle-treated group in the same trial using repeated two-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons test; Error bar=SEM.

Chapter 9 Discussion and General Conclusion

9.1 Model modification

Animal models have historically played a critical role in the exploration and characterization of disease pathophysiology and in the *in-vivo* evaluation of novel therapeutic agents and treatments (697). Especially, genetic technologies boosted the development of high-throughput phenotyping rodent strains to identify the contribution of specific proteins or genes to the pathogenesis of human diseases (698). However, numerous novel drugs that demonstrated promising preclinical effects subsequently failed in clinical trials. This reflects the uncertainty of translating results from animal models to human patients, which in turn raises doubts about their ability to recapitulate all features of complex human diseases and questions their reliability to identify promising treatment options in general (699). Even though three widely accepted criteria, i.e., face validity, construct validity and predictive validity, have been established for decades to measure the ability of animal models to represent symptoms and mechanisms of human disorders, as well as to predict potential therapeutical effects of novel drugs in human trials (423), contradictive results are still reported (460,687). Possible causes for this phenomenon are the complexity of preclinical models *per se*, variances in experimental settings across different laboratories as well as individual differences between strains and animal gender (427). Therefore, detailed modifications of each animal model and the optimization of individual parameters for the specific conditions of a given lab are required to ensure reliability and predictive value of the results obtained.

The physical integration of different tests or models in one single paradigm is thought to minimise the bias of short-term and intra-individual variation in the emotional status of rodents. In this way, the emotional condition of an animal becomes assessable through a series of distinct tasks, contributing to increased reliability and comprehensiveness of behavioural testing (700). In the present study, models of depression, anxiety, locomotion, pain and cognition were integrated as a single paradigm. This was done, to optimize these models to

ensure the validity and synergy of the whole system. This was also done as no systematic utilization of the MCS system in this way has been reported so far. During the initial optimisation stage, we focused on four behavioural models, the light-dark (LD) model for anxiety, the LH model for depression, the NOR for cognitive function and the FE model for PTSD, because of their comprehensive neurobiology and the frequently reported inconsistencies across different laboratories (427,642,701,702). During the period of optimisation, only limited numbers of animals (n = 3) were used to establish the experimental conditions due to financial and ethical consideration. In addition, the results of these optimising experiments perfectly reproduced previously published data (525,703) and consistent results were also obtained from my subsequent experiments that used larger number of animals from different litters. Therefore, neither the use of littermates nor the number of animals influenced the validity of my results.

The LD model is based on the natural preference of rodents to darker areas and their tendency to explore a novel environment. These two conflicting emotions allow to monitor anxiety-like symptoms (704). The advantage of this model is that it does not require prior training or punishment such as food deprivation and shock exposure. Instead, only the natural stressor light is used. This feature enables the integration of the LD model into the LH model, which requires an extensive training phase to induce symptoms by subjecting animals to aversive stressors (635). Typically, rodents spend more time in a dark compartment and this behaviour will increase with increased anxiety levels. Therefore, establishing baseline anxiety levels of rats before exposing them to any type of external stress is essential to evaluate their exploratory activity (705). A wide range of light intensities of 10 to 240 Lux have been reported for using the LD model (566). Thus, a similar range of illumination intensities was tested to optimize the LD model (705). Consistent with previous reports, anxiety levels of non-stressed male SD rats increased with increasing light intensity. In our setting, this model was unsuited to measure

anxiety-like behaviour under stressful circumstances with light intensities above 50 Lux, since the animals failed to explore the illuminated compartment and exhibited maximum baseline anxiety levels. It has to be emphasized that until now, criteria to choose specific light intensities in this model have not been universally established. Based on our data, 15 Lux was used in all subsequent tests, since it represented the brightest tolerated light intensity that did not induce anxiety-like behaviour in rats under normal conditions.

Antidepressant-like effects of drugs can be assessed in the LH model. The typical learned helplessness behaviour occurs when an animal or patient is unable to control repeatedly presented stressful events. After such an experience, they fail to escape from similar stressful situations where their action can stop the stimulus (530). This symptom has also been observed in patients with depression (706). In the LH model, rodents are trained to be helpless by subjecting them to repeated painful foot shocks under inescapable conditions. Their depressive-like symptoms are measured when the animal is subsequently exposed to ES. The LH model was used in the current study because 1) learned helplessness demonstrates high translation across different species including humans and rodents (516) and 2) this model has been widely used in recent years, as it produces reasonably reliable results within a 1-week period, compared to both short-term depression tests (e.g., the FST) and long-term models (e.g., the CMS model) (427). Significant variability has been reported for several key parameters of this model such as number of induction sessions, number of shocks and the length of interphase intervals (535,707). In the optimisation experiments of this study, depressive-like symptoms were successfully induced in male SD rats using parameters that were reported by previous studies (708,709,710), indicating the general reproducibility of this model.

The NOR task for rodents is a non-spatial, non-aversive memory test, which is developed based on the spontaneous exploration behaviour of rats towards novel objects (614). This feature makes the NOR model compatible with the LH model, which in contrast uses aversive stressors

to induce symptoms. Rodents typically respond to environmental changes by preferential exploration of novel objects over those that are familiar, suggesting the formation of memories regarding the identity of objects (711). Due to the relative simplicity and ethological relevance of this model, it was used in the current study to assess drug effects on cognition and to investigate the neural mechanisms underlying learning and memory. In our experiments, recognition memory towards novel objects only lasted for 3 days in the absence of any stress exposure. Parallel to the results of other studies (683,712), stress can result in opposing effects on rats' memory function, which is well demonstrated by our results obtained from two pre-learning stress experiments. Memory is highly dynamic and formed in distinct stages. However, stress can affect all phases of memory formation, including encoding, consolidation, retrieval and extinction, depending on when the subject was stressed (645). If an animal is subjected to stressors before a learning phase, encoding processes can be changed and the subsequent memory formation can be either enhanced or impaired (713). The direction of the effect of pre-learning stress is largely influenced by the interval between the stressful episode and the learning experience (714). If rats are stressed shortly before a learning task, rapid binding of glucocorticoids to non-classical membrane GR facilitates the encoding process. In contrast, if rats are exposed to stress at a considerable period before the learning task, stress can impede new learning and memory processes through a slow glucocorticoid binding to the classic cytosolic GR (715,716).

In the FE paradigm, rodents are initially trained in a FC phase to imprint fear towards a conditioned stressor (CS) (e.g., a tone) by pairing CS with an aversive unconditioned stimulus (US) (e.g., foot shocks). As a result, freezing duration during the CS period, indicative of a fear response, increases. In a second stage, inhibition of fear is obtained by exposing a previously fear conditioned animal to repeated CS in the absence of the aversive US (702). During this procedure, the prediction of CS to the occurrence of US decreases, thus the detected freezing

response during the CS period diminishes over time. In the present study, the fear response of stressed rats only slightly increased during the FC phase, which indicates unsuccessful FC training. This may be a consequence of inappropriate tone intensity (i.e., 68 dB) or frequency (i.e., 10 kHz). In previously studies, the frequency and intensity of tones were generally set as 4 kHz and 75 dB respectively (717). Surprisingly, in the first FE phase of our model, rodents' fears towards the tone continually increased, which was in contrast to the FE theory. This result could have been a consequence of the white light illumination that was used during the FE phase but we did not collect experimental evidence to support this possibility. Previous studies showed that the induction of a novel stimulus can reinstate the extinguished fear response (718). Even though fear responses were not observed in the post-extinction test, the results are not suited to demonstrate the success of the FE training due to of the contradictory behaviour in the FE phase.

9.2 Behavioural effects of opioids in animal models

After the identification of the three classic opioid receptors (719), the behavioural effects of opioids in stress-induced mental disorders were studied in multiple animal models (62). The analgesic potential of different opioid ligands depends on their agonistic or antagonistic activities towards individual opioid receptor types. Furthermore, these ligands also initiate other receptor-dependent behavioural effects such as alterations to locomotor activity, antidepressant, anxiolytic and addictive effects (720). Both marketed and experimental opioids are reported to exhibit antidepressant-like effects in rodent models of depression. These mood-modulating effects are mostly transmitted by activation of the MOP and DOP receptors or by blocking the KOP receptor. (721). On the other hand, there is also some evidence for an analgesic activity of some conventional antidepressants. For example, the tricyclic antidepressant desipramine, exhibits analgesic activity in pre-clinical and clinical trials though

an opioidergic mechanism (722,723). In addition, pain relief associated with the use of some antidepressants may be a secondary effect that involves an interaction of the MOP and DOP receptors (724).

The increasing interest in developing novel opioids as treatments for mental disorders is partially due to the involvement of the opioid system in the complex stress circuitry, with evidence of the release of endogenous opioid ligands in response to stressor exposure (725). For this reason, both endogenous and exogenous opioids are investigated for their potential mood control effects, especially their antidepressant effects. In recent years, antidepressant-like effects of opioid-based analgesics have been demonstrated in variety of stress-based murine models of depression, which also imply the endogenous opioid systems in the pathological mechanism of depression that is based on stress (465,726,727). Opioid receptors widely locate with varying densities throughout the central, peripheral and autonomic nervous system as well as several endocrine tissues and target glands (24). This widespread distribution is consistent with the involvement of opioids in a broad range of functions and behaviors, including regulating pain, reward, stress control, release of neurotransmitters and neuroendocrine modulation (728). For example, decreased expression levels of opioid receptors in the HPA axis was reported in patients with depression, indicating their role in regulating stress circuitry (729). In the past few decades, the close relationship between the opioid systems, stress and the HPA axis has been illustrated in both pre-clinical and clinical studies. Transgenic mice with low level of β-endorphin (an endogenous opioid peptide that predominantly acts as MOP receptor agonist) showed enlarged adrenal glands and deficits in coping with inescapable aversive stress in a depression model (730,731). Furthermore, the MOP antagonist naloxone decreased cortisol secretion in depressed rodents by negatively influencing the release of hypothalamic corticotropin releasing factor (CRF) and inhibiting the HPA axis (731). Moreover, acute morphine administration inhibited the secretion of ACTH at

the pituitary level and decreased plasma cortisol in healthy subjects, indicating an inhibitory effect of the MOP system on the activity of the HPA axis and the stress response (732,733). Similarly, activation of the DOP system also exhibits mood controlling effects. Enkephalins (endogenous peptide DOP receptor agonists) are widely distributed through the limbic system, including the cingulate cortex, hypothalamus, amygdala and hippocampus, where they are mainly responsible for mood control (24). After exposure to a variety of physical and psychological stressors, rats exhibited disturbed mood with evidence of decreased mRNA expression of pro-enkephalin, a precursor of enkephalins in the pituitary gland (734). In addition, the expression of DOP receptors in rats was increased after exposure to chronic stress (735). Apart from chronic stress, exposure to acute stress is able to increase the activity of membrane enkephalinase in the hippocampus. This leads to a break-down of endogenous DOP agonists, which further increased depressive–like symptoms (736). Therefore, the endogenous DOP system seems to directly regulate the stress response and attenuate depressive symptoms. Dynorphins, which have high affinity to the KOP receptor, are widely distributed in the limbic systems and play a critical role in stress-induced mental disorders (737,738). In contrast to the effects of MOP and DOP receptors, activation of KOP receptors in the hippocampus and nucleus accumbens increase the release of CRH from the hypothalamus and hyperactivate the HPA axis, which is associated with the development of depressive symptoms (739,740,741). This is evidenced by, increased levels of dynorphins in the hippocampus after prolonged immobilisation stress, acute swimming stress or the learned helplessness procedure. Such outcomes can be reversed by administration of KOP receptor antagonists (535). Another opioid receptor that is involved in mood regulation is the NOP receptor. This receptor is expressed in various limbic areas and also contributes to the feedback regulation of the HPA axis (742). However, differential effects of NOP receptor system on anxiety and depression have been reported. For example, chronic administration of a NOP receptor antagonist reversed increased

corticorsterone levels in rats under stressful chronic unpredictable conditions (459). In contrast, repeated injections of KOP agonist norciceptin/orphanin FQ (N/OFQ), decreased the expression of anxiety-related behaviors in the elevated plus maze without altering the corticorsterone levels of stressed rats (743). This discrepancy suggests that the modulating effects of the NOP receptor on stress-induced release of corticosterone, as well as its influence on HPA axis activity need to be investigated in more detail.

In our *in vitro* model, the UTAS-synthesised novel bifunctional opioid 1001 simultaneously activates the MOP receptor and blocks the DOP receptor, while in contrast, compound 1003 simultaneously activates both MOP and DOP receptors (unpublished observation, manuscript by A. Paul in preparation). In an *in vivo* study, 1001 exhibited some promising analgesic effects (156) and thus, we hypothesized that the bi-functional opioids might also show antidepressant-like effects due to their selective activities for specific opioid receptors.

9.2.1 Antidepressant-like effect of morphine

Chronic pain and depression are well associated, but the mechanisms underlying the effects of opiates as antidepressants are still unclear. Collectively, there are two possible mechanisms of using opioid-based analgesics as antidepressants. Based on the monoaminergic theory of depression, conventional antidepressants block the reuptake of monoamine neurotransmitters. At the same time, opioids such as morphine also inhibit the uptake of biogenic amines such as noradrenaline and 5-HT (744,745). In mice, a single injection of 20 mg/kg morphine (equal to a human equivalent dose of 1.626 mg/kg based on a bodyweight of 70 kg in human) decreased the content of DA and NA by 23 % and 32 % respectively in the whole brain homogenate (746). In rats, systemically administrated 5 mg/kg morphine (equal to a human equivalent dose of 0.806 mg/kg based on a bodyweight of 70 kg in human) stimulated the striatal dopamine turnover rate in the presynaptic dopaminergic neurons (747). Similarly, in the slices of rat

striatum, 12 nM morphine slowed down the release of DA from the tissue, compared to the non-morphine treated control (748). In contrast, only limited studies are conducted so far to investigate the direct effects of opioid-based analgesics on neurotransmitter levels in human and conflicting results to that of preclinical studies have been reported. For example, a much lower single dose of 10 mg in human (i.v.) (assume the average body weight of participants was 70 kg to give approximately a dose of 0.143 mg/kg, equal to a rat equivalent dose of 0.887 mg/kg or a mouse equivalent dose of 1.759 mg/kg) increased, rather than decreased in rodents, norephinephrine in the cerebrospinal fluid, which was unrelated to the metabolism, synthesis and uptake of the amines (749). Even though, some TCAs such as imipramine convey antinociceptive effects on the other hand by binding to the MOP receptor in both humans and animals (750,751), it is still uncertain if the monoamines play essential role in opioid-regulated pain relief and mood regulating effects due to the above inconsistent results across pre-clinic and clinic studies. Another mechanism is based on the distribution of opioid receptors in the brain according to rodent studies such as amygdala, hypothalamus, thalamus and ventral tegmental area. Since those brain regions control motivation, mood, hormone levels and stress regulation, the expression of opioid receptors in these areas suggest a potential role in mood control (257,752,753). At present, there is some limited evidence that supports the antidepressant-like effect of morphine in the LH model (525,703). However, this effect is still not sufficiently explained due to the described effects of morphine on locomotion, pain relief and cognition, which could interfere with morphine's potential antidepressant activity in this model (754).

To gain a more detailed understanding on the antidepressant-like effect of morphine, as well as to evaluate the validity of our model to produce depressive-like symptoms and detect antidepressants, we assessed the effects of morphine and imipramine side-by-side in our model system. Morphine was administered in line with previous reported regimes with minor

modifications to avoid the development of tolerance (628). In agreement with previous findings, morphine reversed the IS-induced avoidance-escape deficits in our LH model and exhibited imipramine-like antidepressant-like effects. However, at same time, morphine also enhanced avoidance behaviour and locomotor activity in the rats. Furthermore, it showed anxiolytic features in both the LH and LD tests. Because the response to the IS used in the LH model can be influenced by pain perception, emotion, cognition and physical activity (755), the observed antidepressant-like effect of morphine observed in our model are likely modulated by those additional activities of morphine. It can therefore by hypothesized that the antidepressant-like effects of morphine in the LH model can at least partially be seen as artefact. The first artefact is physical activity. Because the determination of depressive-like symptoms highly relies on the physical activity of animals, it is conceivable that higher levels of locomotor activity could improve the avoidance-escape performance of rodents. This possibility is supported by reports that described “antidepressant-like” effects of psychostimulants in the LH model. Those psychostimulants significantly prevented escape deficits by increasing transition numbers during intertrial intervals (756), an effect that was also seen in morphine-treated rats. In the present study, morphine-treated rats exhibited significantly increased physical activity in both the LH model and the OF model. In contrast, in agreement with a previous study, short-term administration of the antidepressant imipramine did not affect locomotion in the OF test (757). Accordingly, our results suggest that the antidepressant-like effects of morphine could be at least partially due to its stimulating effect on physical activity.

Exposure to shocks differentially influences DA activity in the mesolimbic system, based on the current, the duration and the controllability of shocks (758). Foot shocks, over 0.55 mA induced DA outflow in the nucleus accumbens, leading to reduced motivation to interact with the instrumental environment (759). This might partially contribute to reduced escape attempts in the IS group. Additionally, exposure to a mild stressor can increase locomotor activity in the

OF model, while prolonged foot-shock exposure depresses locomotor activity (760). The characteristics of foot-shocks also influence the mesolimbic DA responses. In a previous study, mice exposed to a series of controllable foot shocks show increased DA release in the nucleus accumbens, otherwise decreased DA release in the same region was demonstrated if the shocks were inescapable (761). Conclusively, brief stressor exposure enhances mesolimbic DA release and promotes behavioral activation, but prolonged exposure leads to motional and motivational impairment. Based on the evidence above, inhibition of mesolimbic DA release is more likely relevant in modelling depressive symptoms under laboratory conditions, where severe and prolonged stimuli are usually employed in order to induce depressive-like symptoms (762). Interestingly, activation on MOP receptors stimulates DA neurons in the ventral tegmental area and increases the release of mesolimbic DA. This effect of morphine on regulating neurotransmitter release could contribute to both antidepressant-like effects and increased locomotion observed in our model. In this context, morphine-stimulated locomotion could be considered as dependent on its antidepressant-like activity. Therefore, in current study, we cannot finally establish if the antidepressant-like effects of morphine are only mimicked by increased physical activity or if both of these effects of morphine are intrinsically linked.

The second potential artefact relates to a potential anxiolytic-like effect of morphine. It is conceivable that reduced levels of anxiety could help the animals to escape the stressful conditions, which would manifest in reduced escape failures. In our model, in contrast to other studies (763,764), morphine also showed anxiolytic effects in the LD test after multiple injections, indicating an additional effect relevant to comorbid depression-anxiety disorders. In contrast, no anxiolytic effects of imipramine were observed in any of our models, which is in line with previous studies (765,766). Anxiety exists as two different forms in patients with depression; either as anxiety symptoms or as comorbid anxiety disorders, which are associated with a distinct pathophysiology, treatments and outcome implications (767). Patients with

comorbid anxiety disorders show similar clinical features compared to patients with MDD alone, while pure generalized anxiety disorders show different pathological symptoms (768). For preclinical studies, the LD model is specifically designed to assess anxiety in rodents (568,622) and our results with morphine treatment suggest a role for the MOP receptor in regulating anxiety. This is in line with previous reports in other animal models of anxiety where anxiolytic effects of MOP receptor agonists have been reported (769,770,771,772). The amygdala plays a central role in regulating stress and fear responses and is reportedly involved in the anxiolytic effects of MOP agonists (773,774). While in the amygdala a large number of enkephalin-immunoreactive neurons are found (775), reduced enkephalin mRNA expression in the amygdala was detected in both anxious and/or depressed animals and human patients (776,777). Enkephalins are endogenous opioids that activate MOP receptor, indicating that this receptors could contribute to the stress-regulating role of the amygdala. Therefore, it is not entirely surprising to observe anxiolytic effect of the exogenous MOP agonist morphine in the LD model.

However, in contrast to the LD model, morphine showed no anxiolytic effects in the OF test. Our results demonstrated that the morphine-treated animals spent only a short period of time in the central area of the OF chamber. Even though, increased moving activity was observed in morphine-treated rats, the animals spent the majority of time in the peripheral areas of the OF chamber indicative of higher anxiety-like behaviour. It is worth pointing out that the validity of the OF model to evaluate anxiety-like symptoms and to detect anxiolytics is still questioned, even though this model is increasingly used as a model of anxiety (622). Because the measurement of anxiety symptoms in the OF model relies highly on the physical activity of the animals, psychostimulants could also produce false anxiolytic effects during the OF tests (778). In addition, repeated exposure to the testing environment will also reduce the animals fear towards the experimental environment, which in turn could lead to a higher presence in

the central area of the OF chamber. Hence the OF model still requires significant verification to be used as a valid model of anxiety.

The third artefact is about the potentially increased learning activity of rats in the LH testing phase, which could have been a consequence of the rewarding property of morphine (779). Depressive symptoms are partially characterized by profound deficits in reward-related behaviors such as anhedonia, loss of motivation and memory impairment (780). The brain's reward system, especially the nucleus accumbens (NAc) and the ventral tegmental area (VTA) reward pathway is associated with the development of those reward-related depressive symptoms (781). This VTA-NAc reward circuit is essential for the animals to learn the association between rewarding stimuli and related outcomes (782,783). In addition, this reward circuit also responds to aversive stimuli and is crucial for establishing emotional memory after stress exposure (784). Aversive stimuli were demonstrated to slowly increase tonic extrasynaptic dopamine levels in animals (785), which further inhibit phasic synaptic dopamine release through presynaptic dopamine D2 receptors via negative feedback mechanism (786). In the rat LH model, significantly decreased levels of dopamine-1 receptors and dopamine-2 like receptors in the core and shell of the NAc were only detected in rats that successfully established LH symptoms after shock exposure (787), suggesting a role of mesolimbic dopaminergic systems in mediating behavioral responses to inescapable stress.

The VTA region in rats contains a high density of dopaminergic neurons (60 % of all VTA neurons) and intermediate levels of GABAergic neurons (35 % all VTA neurons) (788). The VTA in the midbrain provides the dopaminergic input to the NAc, which is a target of the mesolimbic dopamine system (789). Activation of MOP receptors in the VTA is important for the rewarding property of morphine (367), which occurs either directly or indirectly (790). In the indirect mechanism, MOP agonists activate VTA dopaminergic neurons by inhibiting GABAergic interneurons and subsequently disinhibiting neighboring dopaminergic neurons

(791). Furthermore, MOP receptor-mediated inhibition on GABAergic neuron in the VTA projects onto cholinergic interneurons in the NAc (792), stimulating dopamine release through nicotinic acetylcholine receptors (793). Given that the VTA contains high concentrations of both MOP receptors and endogenous opioid peptides (794), there is the possibility that MOP agonists can also directly modulate the activity of dopaminergic neurons. This is supported by the preclinical experiments where systemic injected morphine increased the firing rate of dopaminergic neurons (795). In addition, systemically administrated MOP agonists increased dopamine release in the ventral striatum in rats (796). Finally, in an *ex vivo* study, putative VTA dopaminergic neurons were activated by the selective MOP agonist DAMGO (797). Taken together, MOP agonists can lead to increased mesocorticolimbic dopamine release through both dopamine-dependent and -independent pathways, which results in improved locomotor activity and positive (or motivational) reinforcement (798,799). Accordingly, in present study, morphine-treated rats not only showed increased locomotion, but also exhibited better cognitive function or an improved motivational state with increased number of avoidance events. However, it is also possible that the stimulating effect of morphine on locomotion may falsely lead to the interpretation of increased avoidance events as improved learning. Therefore, a significantly more detailed assessment of the effects of opioids on the cognitive function, with regards to learning and memory is needed for future pre-clinical studies to understand the pleiotropic effects of opioids and prevent their misinterpretation.

. . There are two types of learning responses that can be assessed in the LH model: avoidance learning and escape learning. Morphine-treated rats showed more avoidance events, suggesting those rats were better to predict and avoid the incoming shocks. Similarly, imipramine treatment, a drug that has been also reported to improve attention and memory (800), also increased avoidance responses. In contrast, neither morphine nor imipramine treatment had effect on escape learning.

In line with a previous study (801), prior exposure to IS subsequently results in partial interference with the acquisition of instrumental avoidance - escape responses in our model. The interference phenomenon of IS has been shown to be dependent on the shock intensities used. In particular, only high intensities of shocks were able to impair avoidance - escape performance (802). In this study, 0.8 mA and 1.0 mA shocks did not affect escape performance deficits, while 1.3 mA shocks showed a retarding effect on the mean escape latencies. In addition, shock intensities appear to have a U-shaped response curve with regards to escape performance such that shock intensities higher than 1.3 mA reversed the shock-induced escape deficits in that study (803). Surprisingly, 0.8 mA IS in our model only decreased the mean escape latency in the first test but not in the following tests, indicating a short-term facilitating effect on the escape performance. This result is in conflict with previous studies but could be the result of the larger number of shocks that were used in the induction phase of our paradigm compared to other studies.

Escape learning is known to be dependent on the intensity and number of IS (804,805) . In contrast, avoidance learning is not dependent on the characteristics of shocks, but rather represents a prediction of the upcoming shocks. In the LH model, the light and shock stimuli were presented in the form of CS-US pairing. Therefore, the acquisition of avoidance behaviour in the LH testing phase can be seen as a Pavlovian fear conditioning-driven avoidance response, which is more likely a motivational coping strategy of rats to avoid a stressful situation (806).

The fourth artefact is related to the analgesic effect of morphine, since a higher level of escape performance could be a consequence of decreased morphine-mediated pain sensitivity. Stress-induced analgesia (SIA) is a physiological response to aversive stressful events that also effectively suppresses pain through either the production of endogenous opiates (endorphins) (668) or by a non-opioid mechanism that stimulates the release of endogenous cannabinoid compounds in the periaqueductal grey matter of the midbrain (807). Assessing this response

using animal models/tests can help to elucidate the fundamental mechanisms of nociception, as well as potential therapeutic targets for pain and stress-related disorders. Inescapable foot shocks used in the LH model were previously reported to induce SIA if the number of foot shocks exceeded 60 and the shock currency was over 0.55 mA (653). Since this study employed 90 IS at 0.8 mA in a single induction session for two consecutive days, the foot shocks were possible to evoke opioid-based SIA. The development of SIA in the LH model parallels clinical observations of increased endorphin concentrations in the cerebrospinal fluid of patients with MDD (808). In support of this, we observed and increased TF latency 24 h after the first induction, which could indicate the release of endogenous opioids in response to the stress. It is interesting to note that in our model the nociceptive responses measured by the TF and HP tests showed different results, which is likely a consequence of what these two assays actually measure. While the TF test detects radiant heat evoked spinal reflex latency and the HP test measures a contact heat evoked supraspinal response (638). In the HP test only a non-significant trend towards an increased nociceptive response was observed, which suggests a predominant spinal controlled effect of IS on nociception in our model. In line with a previous study (809), our results also demonstrate that the SIA lasts only for a short period of time since an increased nociceptive response was not observed at the end of LH experiment.

It is important to note that the administration of morphine in our model occurred at a time when SIA was no longer present. This was necessary to distinguish the source of altered nociception. Because morphine was only administrated after shock exposure and nociceptive response was only measured at the end of LH experiment (Day 6), no morphine-induced effects on thermal-induced nociception were observed. Based on these result and considerations, we conclude that the analgesic effects of morphine did not interfere with the escape performance of our test animals.

Another noticeable effect of morphine was related to body weight changes. In this study, foot-shocks did not have any effects on body weight when compared to the NS group. The results of the present study are in line with a previous study (683), while significantly decreased body weight was reported in another study (810). These inconsistent effects may be due to differences in the protocols used, such as the length of the foot-shock period and the strength of the shocks. In contrast to the IS and NS groups, 2 injections of morphine resulted in a significant increase in body weight in stressed rats (IS+morphine). Two-way ANOVA analysis indicated that both the procedure and treatment *per se* affected the detected body weight increase. Therefore, two explanations for the significant body weight changes in the morphine-treated group are possible. Firstly, stressors in the LH model have been reported to induce taste aversion in SD rats, which subsequently reduce food intake, which could negatively affect weight (811). Based on my results, IS may only affect the appetite of the animals in the short-term, because in the IS group, slightly increased body weight was observed from day 2 onwards. Therefore, beyond day 2 when the IS were stopped, morphine-treated rats might have increased food intake. Secondly, morphine treatment could reverse the IS-induced emotional impairment due to its rewarding effects (812), which subsequently could have resulted in increased food intake and body weight. However, a direct evidence for the influence of morphine on weight gain in rodents has not been demonstrated so far. In addition, excessive plasma glucose is linked to increased body weight (813). However, morphine has been demonstrated to stimulate insulin release from pancreatic beta-cells in culture (814), which does not support a morphine-induced weight gain after multiple injections.

Unexpectedly, in the imipramine-group, the rats showed a significant drop in body weight on day 2 when the animals had not actually received any imipramine yet. This could have been the result of individual differences in the stress response of the animals in this group (815), where some rats of this group were more sensitive to the negative emotional impact of the foot-

shocks. This could indicate that the results from the imipramine group are not necessarily directly comparable to the IS and stressed morphine-treated groups due to animal differences. However, since the imipramine-treated group showed responses in line with the majority of previous reports (683,816,817,818,819), this difference in group composition, if present at all, might not be very significant for the overall conclusions of the project.

9.2.2 Antidepressant-like effects of bifunctional opioids

The opioidergic systems play a central role in pain management, which is mainly transmitted via the activation of the MOP receptor (266,820). Over the past decades, morphine has also demonstrated that it can improve the mood of patients with chronic pain and cancer (821), suggesting a general potential of MOP agonists in mood control. However, MOP activation also induces reward behavior, which contributes to its addictive properties (822). In addition, long-term use of MOP agonists leads to the development of analgesic tolerance, which reduces the effectiveness of pain control (823). Therefore, the pain-killing and behavioural properties of other opioid receptors and their corresponding ligands are currently investigated by several groups to identify alternatives with less side effects compared to morphine. Recently, DOP receptors have gained significant interest due to their dual activities around analgesia and mood regulation (289). Focusing on depression, compared to wild-type animals, DOP receptor knockout (KO) animals exhibited anxiety-like and depressive-like symptoms. This feature is mirrored by the DOP antagonist naltrindole that was able to block the antidepressant-like effect of DOP receptor activation in the LH model (531). Based on this evidence, strategies to design MOP/DOP-specific bifunctional opioids are underway. Compared to ligands selective for a single receptor, these molecules promise to display improved efficacy and reduced side effects (368).

Two novel bifunctional opioids have been synthesized at the University of Tasmania and were named 1001 and 1003. While 1003 activates both MOP and DOP receptors (unpublished observation), 1001 activates only the MOP receptors but acts as an antagonist for the DOP receptor (679). There is some uncertainty about the *in vivo* toxicity of 1001, since conflicting effects have been reported (682,824). In contrast, the safety profile of 1003 has so far not been evaluated. In our study, we aimed to compare the effects 1003 on cellular ATP levels *in vitro* as surrogate marker for cellular toxicity (825) against 1001. Over a 24 h incubation period with 1003, no overt toxicity was detected, compared to 1001, morphine or untreated cells. In 1001-treated cells, however, significantly decreased ATP levels were detected after 24 h *in* vitro, suggesting the presence of metabolic toxicity. This outcome was in line with previous reported result by another group (824). However, this result is in contrast to a previous study where 1001 was used systemically in rats over 5 days and no toxic effects were reported (682). The demonstrated absence of *in vivo* toxicity may likely be because drug toxicity was not assessed further in sufficient detail in tissue specimens that are susceptible to energy impairment such as heart, skeletal muscle or retina. In addition, under physiological conditions organs differ with regards to metabolism and detoxification of xenobiotics from cells *in vitro*, which can lead to differential metabolic and toxicity outcomes. Nevertheless, energy-impairment-induced toxicity *in vivo* typically leads to a delayed phenotype after weeks of drug exposure (826). Thus, 1001 was still tested in animal models based on the reported short-term safety profile in rats (682), even though it remains necessary to re-evaluate the safety of 1001 in much more detail over longer time periods in future studies.

In our models, 1001 and 1003 failed to affect depressive-like symptoms, anxiety-like symptoms, nociception or locomotion. There are several possible reasons for this observation, which could be related to the route of administration and the dosing of both drugs. The previously reported analgesic effects of 1001 were obtained using intrathecal injection (682),

a method that circumvents the blood-brain-barrier. Therefore, one explanation for the lack of behavioural changes could simply be that insufficient drug reached the central nervous system. This hypothesis is somewhat supported by the observation that 1001 and 1003 at higher concentrations with subcutaneous injection do show some limited analgesic effects (unpublished observation by PhD candidate A. Paul).

In addition, based on the enhanced avoidance behaviour due to morphine treatment, we hypothesised that the two bifunctional opioids might also positively affect cognition based on their MOP agonistic activity. To support this hypothesis, we measured the cognitive effects of 1001 and 1003 treatment using the NOR model in rats that were subjected to the LH paradigm. In the NOR model, animals of the IS group showed both impaired short-term and long-term recognition memory, as previously documented (827). In rats treated with 1001 and 1003 a clear trend towards improved memory was observed that however did not reach significance.

The HPC is considered to be one of the main brain centers involved in mood regulation and it is also highly sensitive to stress (828). We therefore measured the density of oxidatively damaged cells in the granule cell layer of the hippocampal DG region. Surprisingly, 1001 and 1003 demonstrated a trend towards reduced oxidative cellular damage compared to the IS group similar to the trend described above towards improved memory function. Exposure to IS can induce the production of ROS, which can lead to oxidative stress (829), lipid peroxidation (830) and HPC-related cognitive dysfunction (831). The DG is the hippocampal region where neurogenesis happens during adulthood (832). In particular the granular layers of the DG contains large number of granule cells that were mostly generated after birth (833). Those adult-born dentate granule cells (DGCs) heavily contribute to learning and memory and adverse experiences during a critical period can influence survival and activity of these cells, which can have a direct impact on cognitive function (834). The IS used in the LH model have been reported to decrease cell proliferation and neurogenesis in the DG of adult rodents (835),

which could explain the impairment of learning and memory observed in the IS-exposed rats in our study. In addition, stress exposure can also decrease plasticity of the DG, which would influence the quality of the formed memory (836). This “blurry” memory is thought to last for only short time periods (837), which would explain the memory performance differences of stressed rats 30 min and 24 hours after the learning phase in the preliminary study.

MOP and DOP receptors are widely expressed in the granule cell layer of the DG (838). There is some evidence that blocking MOP receptors can enhance memory in mice (839) and activating DOP receptors can improve plasticity and excitability of the DG (840). However, in this study, 1001 and 1003 showed similar trend towards reducing oxidative stress in the hippocampus, while 1001 seemed to be more effective in enhancing memory compared to 1003. But given that 1001 and 1003 show no analgesic effects after s.c. injection at the doses used in this study, it is questionable if these compound can even enter the brain (841). In this context it is, therefore, difficult to hypothesize how activation/inhibition of the MOP and DOP receptors could have affected cognitive function in the present study. In previously studies, MOP knockout mice were associated with reduced cognition in a NOR model (842), which would suggest that activation of the MOP receptor could enhance memory. In support of this hypothesis, a single systemic injection of morphine reversed stress-induced memory loss in rodents (843). However, improved memory in MOP knock out mice in a hippocampal-dependent spatial memory task was reported previously (844). In this study, the authors speculated that this effect was transmitted by increased proliferation of adult-born DGCs. Similar inconsistent results were also reported for the DOP receptor system. Both a selective DOP agonist (845) and DOP antagonist ICI 174,864 (846) were reported to enhance memory function in mice after a single administration. Therefore, the specific effect of the MOP and DOP systems on cognition are still largely unclear due to the inconsistent results reported across different labs. Additionally, opioids produce a wide range of behavioral effects, and it

is not known to what extent the observed influence (either positive or negative) on cognitive processes in animal models are directly mediated by a specific opioid system or results indirectly from other behavioral effects of opioid ligands. As discussed above, activating the MOP receptors can increase the release of mesocorticolimbic dopamine, thus improving physical and emotional states of rodents (798,799). Taken the NOR model as an example, it is possible that injection of 1001 and 1003 might have increased locomotion, which would have resulted in increased exploration activity overall, with the potential to produce false positive results interpreted as cognitive enhancement. Together with the effects of 1001 and 1003 on oxidative stress damage in the HPC, it would be beneficial to increase animal numbers and apply higher doses of both drugs in future studies to increase potential brain levels. This approach might lead to significant antioxidant effects and significantly improved recognition memory. Since administration of 1001 and 1003 occurred during all stages of memory formation, i.e., encoding (object learning), consolidation (interphase interval) and retrieval (memory test using novel objects) (642), our study was unable to pinpoint specific stages of memory formation where the bifunctional opioids might be active. Thus, it would be helpful to apply different injection regimes to investigate the specific influence of 1001 and 1003 at different stages of memory formation if the experimental conditions allow this approach.

9.3 Monoamine oxidase and reactive oxygen species

Elevated MAOA density and activity, which directly result in reduced monoamine levels, are detected in a variety of brain regions (e.g., cingulate cortex and hippocampus) in patients with depression (308,847). Furthermore, in most cell types MAOA localizes to the outer mitochondrial membrane (848). As a natural product of MAOA enzymatic activity, the cytotoxic product hydrogen peroxide (H_2O_2) is formed (849,850). In support of this, the nonselective MAO substrate tyramine induced a dose-dependent cellular ROS production. The

observed selectivity of dyes that were able to detect this ROS production in our cells suggests that the produced H_2O_2 was mainly localized to the cytoplasm and not to any membrane compartments.

MAO-generated H_2O_2 can be partially converted into highly toxic hydroxyl radicals such as $OH^{\bullet}$ through reacting with ferrous iron (851). This and subsequent follow up reactions will lead to increased levels of oxidative stress which can result in widespread oxidative damage to cellular macromolecules. As a consequence, increased oxidative stress can disturb the HPA axis function, which is involved in the development of depressive symptoms (852). In our experiments, both 1001 and 1003-treated rats showed a trend towards reduced oxidative cell damage in the hippocampal DG and slightly improved recognition memory. We therefore hypothesised that opioids might enhance cognition by decreasing oxidative damage. In this context, it is possible that mitochondrial MAOA, as a known source of oxidative stress, may be instrumental in the mechanisms of depression-mediated cognitive improvement.

Currently, there is some evidence for a mito-protective effect of DOP agonists *in-vitro* that is associated with an ability to reduce cellular levels of oxidative stress (624,853). However, there is no direct evidence that demonstrates that mitochondrial MAOA expression is the target of opioid-mediated reduction of oxidative stress, which could account for the neuroprotective effects of opioids. Therefore, we attempted to measure changes to MAOA expression before and after IS exposure and 1001 and 1003 treatment. In the WB analysis, multiple bands that ranged between 170 and 55 kDa were detected with no differences across different tissues. These bands were also not affected by tissue processing methods and brain regions used. Since there is considerable confusion about the expected molecular weight of MAOA by different antibody manufacturers, we aimed to establish the expected protein size of MAOA (rat). For this purpose, we used three different online calculation tools based on the MAOA protein sequence obtained from NCBI protein database (**Figure 83**)

(https://www.ncbi.nlm.nih.gov/protein/EDL97663.1).

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      421 rvirqpvgri yfagtetatq wsgymegave ageraarevl nalgkvakkd iwveepeskd
      481 vpaieithtf lernlpsvpg llkitgvsts vallcfvlyk ikklpc
```

Figure 83. Protein sequence of monoamine oxidase A of Sprague-Dawley rats.

The calculated protein size of MAOA is approximately at 59.5 kDa (**Figure 84-86**), in accordance with the reported size in the GeneCard database (59.7 kDa, http://www.genecards.org/cgi-bin/carddisp.pl?gene=MAOA&keywords=MAOA).

Tool 1: Protein Molecular Weight (http://www.bioinformatics.org/sms/prot_mw.html)

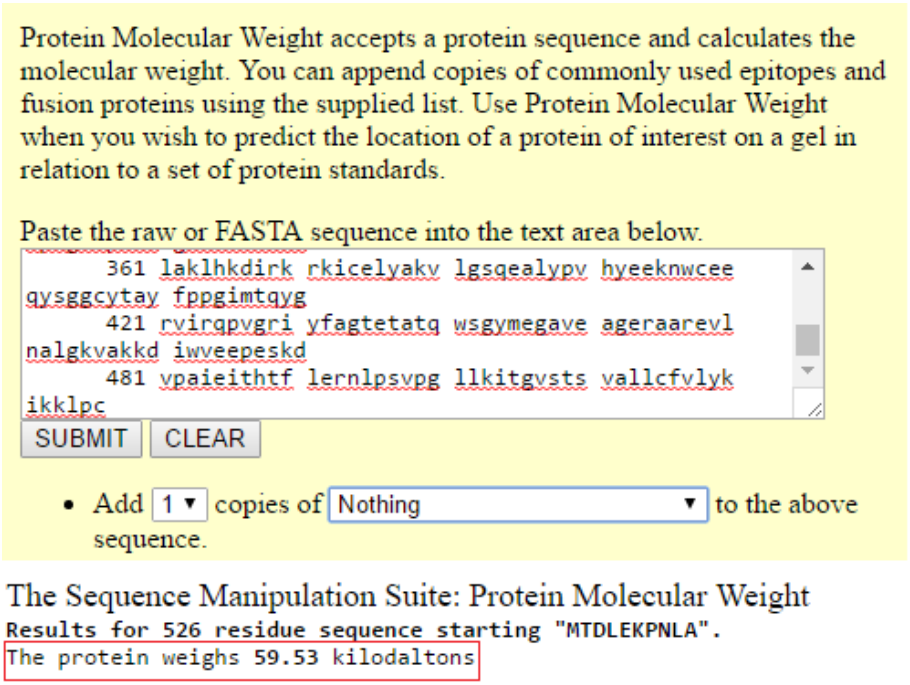

Figure 84. The protein weight of the monoamine oxidase A of Sprague-Dawley rats calculated with Protein Molecular Weight.

Tool 2: Peptide Molecular Weight Calculator –GenScript (https://www.genscript.com/tools/peptide-molecular-weight-calculator)

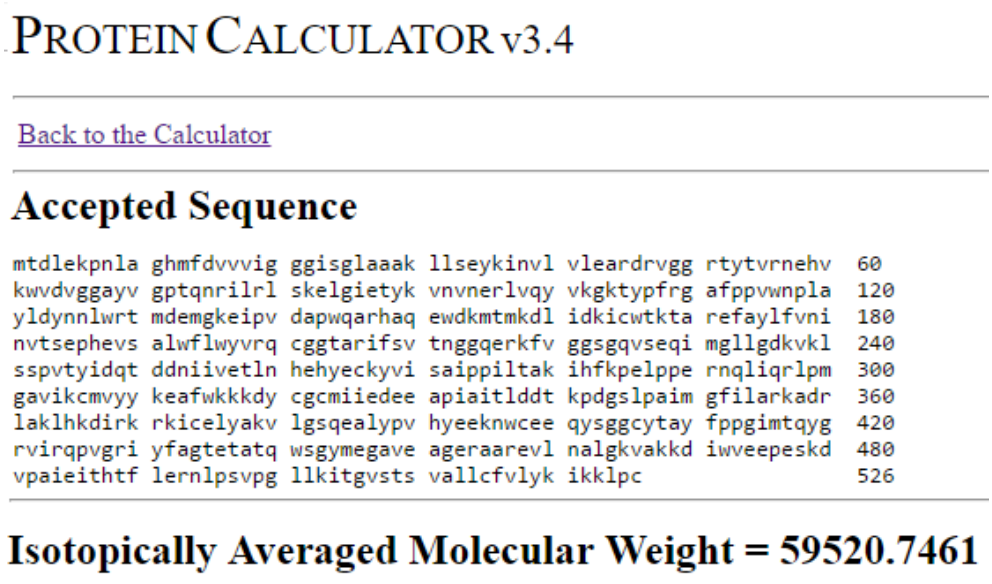

Figure 85. The protein weight of the monoamine oxidase A of Sprague-Dawley rats calculated with Protein Calculator v3.4.

Tool 3: ExPASy – compute pl/Mw tool (http://web.expasy.org/compute_pi/)

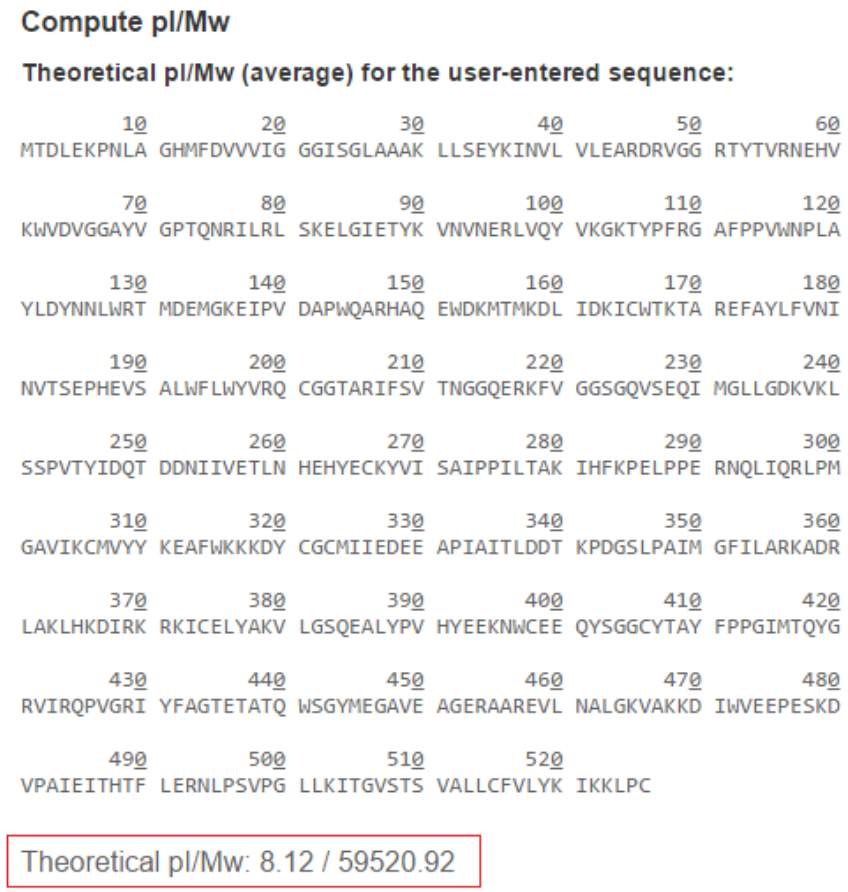

Figure 86. The predicted molecular weight of rat mitochondrial monoamine oxidase A calculated using ExPASy.

The fact, that we detected a single band of the expected size for MAOA in the rat liver microsomes that were used as positive control suggests the appropriateness and function of the used antibody and our staining methods. Therefore, one explanation for our inability to detect MAOA in the rat brain could be that MAOA in this tissue is heavily post-translational modified (PTM) through N-acetylation (854), ubiquitination (855) or phosphorylation (856), which would affect its apparent size on a western blot. On the other hand, we cannot exclude that MAOA might be exquisitely sensitive to proteolytic degradation in brain tissues and that in our experiments MAOA was lost by degradation during dissection or the preparation of brain lysates. Finally, another possibility is that MAOA is expressed in brain tissue as alternative splice variants of different molecular sizes. However, this possibility is not reported in the

literature and we were unable to find any evidence for this in several databases (i.e. Genecards). Consequently, we had to conclude that western blot analysis is not suitable for the current study. Therefore, we addressed the question by using immunofluorescent staining of tissue sections. While a strong fluorescent signal, reminiscent of axonal mitochondrial MAOA-specific staining was observed, no differences were detected in the HPC of the NS and IS treated animals. There is a possibility that MAOA activity rather than its expression could be increased in patients with depression (857). Therefore, the potential antioxidant effects of 1001 and 1003 in rat brains might be due to a direct or indirect inhibitory effect on MAO activity or additional uncharacterized ROS-producing systems. Another possibility for this effect could be a opioid-dependent regulation of the HPA axis, which also could reduce the levels of oxidative stress (858). A final possibility for the observed slight anti-oxidative activity of 1001 and 1003 lies in their structure. These peptidomimetic opioids share a significant amount of structural similarity with so called SS-peptides (named after their inventors) that all contain the characteristic di-methyl tyrosine (DMT) moiety (859). Like the test compounds 1001 and 1003, these SS-peptides also originate from opioid-receptor ligands but have been described as potent antioxidative compounds due to its DMT moiety and have shown promising mitoprotective and neuroprotective activity *in vivo* and are now in clinical development (860). However, since we did not assess the plasma concentrations of cortisol, MAO activity or a direct antioxidative activity of 1001 or 1003 in the present study, it is not possible to judge, which potential mode of action was the underlying reason for the observed trends.

9.4 Behavioural effects of idebenone

In this study, 200 mg/kg idebenone was tested for its behavioural effects and antioxidant property under stressful conditions. In previous studies, idebenone has successfully demonstrated neuroprotection of retinal neurons in animals at a dose range of 200 to 2000

mg/kg (629). In addition, 100 mg/kg idebenone was proved to distribute widely in brain regions of the temporal and occipital cortices, nucleus accumbens, hypothalamus, hippocampus, striatum and cerebellum after oral administration using a radioactive labelling technique by ^{14}C (861). Given the similarity between the blood-retina and blood-brain barrier penetration of drugs (630), 200 mg/kg idebenone is sufficient to reach the brain and the use of this dose was fully justified and accurate.

Cognitive effects

Based on the behavioural and histological data of 1001 and 1003, we postulated that the antioxidant effect of a drug could be crucial to their antidepressant-like effect in our animal model of depression. To verify this hypothesis, we aimed to employ a much more potent antioxidant in our models. Idebenone is a synthetic structural analogue of coenzyme Q and has demonstrated antioxidative, mitoprotective and neuroprotective properties in multiple models (862). Currently, it is used to improve cognitive function in some dementia patients (694) and has been recently approved for the treatment of a mitochondrial disease by the European Medicines Agency (EMA) (384). It is thought that the reported neuroprotective effects of idebenone are partially due to its antioxidant effect via inhibiting cellular lipid peroxidation (863). In line with this well described antioxidative activity, idebenone treatment over both 3 and 7 days reversed the oxidative cell damage in the anterior HPC that was caused by the IS exposure in our study. In the NOR model, untreated rats showed memory deficits due to IS exposure. According to the histological analysis, this resulting stress-induced cognitive dysfunction was associated with oxidative cell damage in the granule cell layers of the DG area, which is essential for learning and memory. In support of our hypothesis, idebenone not only reduced oxidative damage but also reversed the memory loss in our model. However, this cognitive effect of idebenone in the NOR model was only observed after 7-day administration,

even though significant reduction on oxidative cell damage was also detected after 3-day treatment. Because our study did not assess cell proliferation in the DG, the effect of different treatment periods with idebenone on neurogenesis in the context of recognition memory is unclear. It is also notable that significant idebenone-dependent reduction of oxidative damage was only detected in the anterior HPC. There is still some controversy over the exact function of the anterior versus the posterior HPC. Some studies argue that the anterior HPC processes primarily stress, emotion and affect, while the posterior HPC is heavily involved in cognitive function, such as spatial memory (864). In our model, IS caused similar levels of oxidative cell damage in both HPC regions, which suggests an overlapping role of both hippocampal regions in stress response. However, based on our results, the anterior HPC appears to play the major role in processing non-spatial information, such as recognition of different objects at the same location in the NOR model.

Antidepressant-like effects

In our study, idebenone showed no effects on depression, anxiety, food intake/body weight or locomotion, and clearly showed the independence between the antioxidant and antidepressant-like properties of idebenone, which significantly questions the general use of antioxidants as antidepressant alternatives (689). However, since our rats only received idebenone treatment for up to 7 days, we cannot exclude the possibility that longer treatment periods with antioxidants could still have some antidepressant effect. To explore this aspect appropriately, long-term studies with multiple potent antioxidants in animal models of long-term stress exposure, such as the CMS model, would be more appropriate.

Pain

Currently, analgesic effects of antioxidant supplements have been reported in patients with chronic pancreatitis and rheumatoid arthritis (865,866). Since idebenone is a potent antioxidant, we hypothesized that idebenone might also have some analgesic effect. In fact, there is some limited evidence that idebenone could indeed have some intrinsic analgesic activity. In a study that looked at inflammation and pain in a rat model of carrageenan-induced inflammatory pain, the authors described some "anti-inflammatory" activity of idebenone (867). However, the nature of the model system was unable to differentiate if the protective effects were due to anti-inflammatory or analgesic properties of idebenone (867). When we measured the pain threshold of idebenone-treated rats in the TF test, we did not observe any changes to the pain perception in our test animals. However, this result might be due to the relatively short treatment period of this study as previous studies, showed analgesic effects of antioxidant supplements only after weeks or even months of treatment (865,868).

Fear extinction

To confirm the effect of idebenone on cognitive function under stressful conditions, we additionally employed the model of fear extinction (FE), which is widely used to assess the information processing ability after stress exposure (646). The FE model is closely related to the commonly used exposure therapy for anxiety disorders, such as PTSD (869). This cognition-based therapy involves exposure of patients repeatedly to their feared objects without real exposure to danger, in order for the patient to overcome their fear to the stimuli (870). It has to be stressed that clinically used FE learning is based on an active learning process to separate the connection between a traumatic event and possible triggers. Given that PTSD patients are under chronic stress, their ability to learn is significantly reduced and hence FE learning is reportedly only effective in a small group of PTSD patients (871). Therefore, pharmacological interventions that are able to enhance learning and memory could be improve

success rates of FE therapy treatment. Based on the results of idebenone in the NOR model, we hypothesized that idebenone could enhance the active learning process under stressful conditions, hence, inhibiting the return of fear. For this purpose, we first needed to modify the model of FE using the MCS. Unexpectedly, an increased fear response was detected during two FE sessions in the model-optimization experiment. The white-light illumination used during FE training could have acted as a novel stimulus and to enhance the fear of our test animals towards the shocks (871). In addition, the use of a grid floor in the FE phase may have also reminded the rats of the aversive shock, thus inducing fear to the experimental environment. Therefore, in the idebenone experiment, we applied three major strategies to improve the validity of our FE model. First, we adjusted the length of shocks and tones in order to enhance the strength of fear conditioning (FC) training. We observed that the presence of the grid floor initiated a freezing response as soon as they were placed into a new environment that has a grid floor. Therefore, as a second measure, to distinguish the influence of shocks and environment on freezing behaviour, a solid floor instead of a grid was used to increase the discrimination between the chamber in which rats have received shocks and the chamber without shocks. Finally, the same lighting conditions were used for all phases, in order to avoid inducing extra stimuli that could produce more fear.

When we tested idebenone in this model, we used the same 7 day pre-treatment that showed significant effects in the cognition model. However, this meant that idebenone was already present during the FC phase. This was an experimental problem, we could not avoid at this point, as shorter exposure times with idebenone would have likely shown no response. Therefore, we were only partially surprised that idebenone-treatment in the modified FE model produced significantly increased conditioning (= increased fear response) compared to the untreated group. It appeared that the idebenone-treated animals after only 2 rounds of conditioning had already learned the connection between sound and shocks, while the untreated

animals required significantly more sessions. In fact, in the FE phase the untreated animals showed only a low fear response, which suggests insufficient memory consolidation. In contrast, the idebenone-treated animals showed a significantly better fear response in the first round of FE, reflective of a more efficient learning process during the FC phase, which on the one hand, supported our hypothesis that idebenone can significantly improve learning behaviour under stress. Unfortunately, on the other hand, this meant that the FE experiment was unable to provide us with the answer we required as the two groups (treated and untreated) did not start with the same level of anxiety at the start of the FE paradigm. To overcome the insufficient FC in the untreated animals a longer interphase interval between FC and FE phases could be more appropriate for future studies. In fact, there is some evidence that the FC response might persist for 14 days (872), which suggests that idebenone treatment for 7 days after the FC training phase might be able to provide sufficient memory consolidation, and also restrict drug effects to the extinction learning phase.

9.5 General conclusion

9.5.1 Limitations of this study

This study investigated the psychopharmacological effects of opioids and antioxidants in a complex integrated model of different behaviours to provide a possible explanation for the relationship between oxidative stress, depression and depression-related cognitive deficits. Although this study addressed all the initial aims and objectives, there are limitations that have to be considered.

In the *in vitro* study, we confirmed that activating MAOA induces ROS. However, this study did not demonstrate any effects of opioids or idebenone on MAO activity in the ROS assay. Hence, we are not sure if opioids and mitochondrial-based drugs can influence MAOA activity

in term of ROS production. In addition, we did not establish a real dose-effect relation for the toxicity of morphine and bifunctional opioids *in vitro*.

In the *ex vivo* assays, we only showed that the levels of MAOA were not affected by exposure to inescapable foot shocks, which is in contrast to increased MAOA activity and expression levels reported in patients with MDD (873). This discrepancy has never been reported for the LH model before and although it points to a major limitation of the depression model used in this study, it also highlights that this problem has to be investigated for other pre-clinical models of depression to judge their predictive value. This leaves the possibility that in our model not MAOA expression was altered but its activity. In addition, this study noticed a potential uncertainty around MAOA antibody specificity when used for western blotting. This could either have been the consequence of too little MAOA protein in total brain extracts or a true problem with antibody specificity. This issue could have been addressed by testing the antibody on rat neuronal cultures *in vitro*. For this purpose in isolated brain cells, MAOA expression could have been modulated *in vitro* using either overexpression and/or antisense/siRNA technology, which could have confirmed antibody specificity but was not used in the current study. Therefore, this omission has to be regarded as a potential limitation of the current study. Furthermore, it is established that mitochondrial ROS is generated by MAOA-dependent metabolism of catecholamines as well as the mitochondrial respiratory chain (874). However, we did not directly assess mitochondrial ROS production or tried to establish which oxygen radicals were specifically produced during the state of LH-induced depressive symptoms as in vivo ROS measurements still presents with nearly unsurmountable technical difficulties. We also did not specifically assess HPA function. Therefore we have no causal proof for our hypothesis that oxidative cell damage is associated with mitochondrial dysfunction and HPA axis dysfunction. In this context, future studies are needed to investigate

the effects of IS and drugs on neurogenesis in the hippocampal DG, in order to identify the possible underlying mechanisms of oxidative stress-related memory deficits in depression.

In the *in vivo* study, the experiments were not performed in a blinded manner. Since I was not working within a team and was responsible to look after the animals, perform all treatments and the daily behavioural testing on my own, this an unavoidable limitation. However, all calculations and scoring processes were automatically performed by the acquisition and analysis software of the Multiple Conditioning System based on investigator defined parameters. These parameters were informed by reports described in the literature (875) and the manufacturers recommendations and were kept constant for all experiments. This approach was seen to effectively minimize investigator bias. Nevertheless, investigator blinding to the treatment groups could have increased quality of the data and should be considered for future testing. In addition, the discrepancy in animal body weights between different groups, especially between the control group and the treatment groups of the morphine experiment may potentially have affected the results of the drug-treatment experiment. It is possible that body weight changes are a result of alterations of hormonal status (876). On the other hand, higher body weight has been found to result in larger pituitary and adrenal glands in male rats (877). Since these two hormone glands are involved in the regulation of stress hormone secretion (878), stressed rats with larger body weight might show more EFs. In addition, a recent study reported that rats with higher body mass showed more exploratory behaviour (879). Based on this evidence, hypothetically rats in the NS and IS groups of the morphine-imipramine experiment might exhibited more movement in the OF test due to their large body weight. However, in my study, neither more EFs nor increased physical activity was observed, when compared to the control groups of the idebenone experiment. Therefore, I believe that this potential limitation was not of major concern in the present study.

Another limitation is about the effect of morphine on learning and memory. Morphine improved avoidance learning in rats, but this cognitive effect was not assessed in the NOR model in contrast to the cognition effects of idebenone that were tested in this model. This makes a direct comparison of the effects of both drugs impossible based on the variability seen in the results from both cognition models. Due to the sequence of experiments in this project and the preparation of brain samples of morphine treated rats for western blotting, we did not measure effect of morphine on oxidative cell damage in the HPC either. Therefore, we cannot confirm that the effects of morphine on learning and memory are related to ROS and oxidative damage in the HPC similar to the effects of the novel bifunctional opioids. We also have to acknowledge that we did not test higher doses of both bifunctional opioids 1001 and 1003 in the LH model. This was mainly a consequence of the limited availability of compound for in vivo studies as these compounds involved a difficult synthesis and hence we did not have access to unlimited amounts. Nevertheless, as a consequence, we are unable to confirm or refute that higher concentrations would have resulted in any antidepressant effects comparable to morphine. Unfortunately, we were unable to show if idebenone improves fear extinction in the FE model, which was on the one hand a problem of the experimental design with regards to the idebenone pre-treatment period and also the consequence of time restriction to further improve the model. Hence, whether idebenone could indeed be used to facilitate extinction learning will have to be addressed in future studies. Due to this time restriction, we were also unable to test different doses and treatment periods of idebenone to establish a possible time and dose-dependency. Overall, this study now serves as the basis for future trials to optimize experimental conditions and to generate detailed concentration and time-dependent data that are necessary to potentially translate our results into a clinical setting.

9.5.2 Summary

Based on to the distribution and function of different types of opioid receptors and their specific endogenous ligands/drugs, previous studies widely proposed to develop novel opioids for mood control (282,724,725,777,880). This project was initially developed based on those points of view. On the basis of the reported antidepressant-like effects of morphine in the LH model (525), we established and verified an integrated model by evaluating the effects of morphine on different interference factors of the LH model. In line with previous results, morphine-treated rats exhibited promising antidepressant-like effect in our model. However, this study was able to show that at least part of this activity could be an artefact based on the anxiolytic, physical stimulating and cognitive effects of morphine.

The results of initial tests of morphine and imipramine against depression nevertheless led us to investigate two novel bifunctional opioids. In our laboratory, those two bifunctional opioids 1001 and 1003 showed affinity towards both MOP and DOP receptors. Since the toxicity *in vivo* of both novel opioids are uncertain, we tested both drugs in regards to cellular bioenergetics and proliferation. Surprisingly, we found that 1001 which activates the DOP receptor was toxic to liver cells, which was contradictory to previous report (881). However, the same drug entity has been previously tested *in vivo* and while one study reported no toxicity (682), there was some information about toxicity reported later (824). Although both bifunctional opioids showed no effects on depression, anxiety, pain and locomotion, both 1001 and 1003 showed a slight non-significant reduction of oxidative damage in the HPC (**Figure 6**). At the same time both drugs also showed a trend towards improved memory function. These results highlight a link between oxidative stress and depression-associated memory loss. Based on this potential connection, we tested the psychopharmacological effects of the potent antioxidant idebenone in the same models. Similar to the bifunctional opioids, idebenone did not have any effects on depression, anxiety, pain or locomotion, but it ameliorated oxidative

damage in the HCP and effectively reversed stress-induced memory deficits. This strongly suggests that oxidative damage in the hippocampal DG presents a direct cause of cognitive deficits in depression.

Mitochondrial MAOA, as one of the main targets for current anti-depressant therapy, not only directly contributes to the decrease in synaptic catecholamines that is characteristic of MDD (294,308), but is also a source of ROS (882) (**Figure 6**). MAOA generates H_2O_2 during the metabolism of catecholamines, which is further converted into a varierty of oxygen radicals (883) that damage to cellular macromolecules and lead to mitochondrial dysfunction, which represents a vicious cycle that exacerbates oxidative stress (884) (**Figure 6**). ROS and mitochondrial dysfunction are highly associated with the development of mental disorders and neurocognitive deficits (882). Before this study, it was unclear if ROS are causally involved in the pathogenesis of the MDD and its related memory loss or if ROS represent an indirect consequence of depression (885). The results of this study now highlight that antioxidant strategies are unlikely to be effective against depression but have a clear potential to alleviate the depression-associated cognitive impairment. Therefore, antioxidant strategies are likely to be developed in the future as novel treatment paradigms against depression-associated cognitive dysfunction but also for additional indications that are associated with impaired cognition under stressful conditions (**Figure 87**).

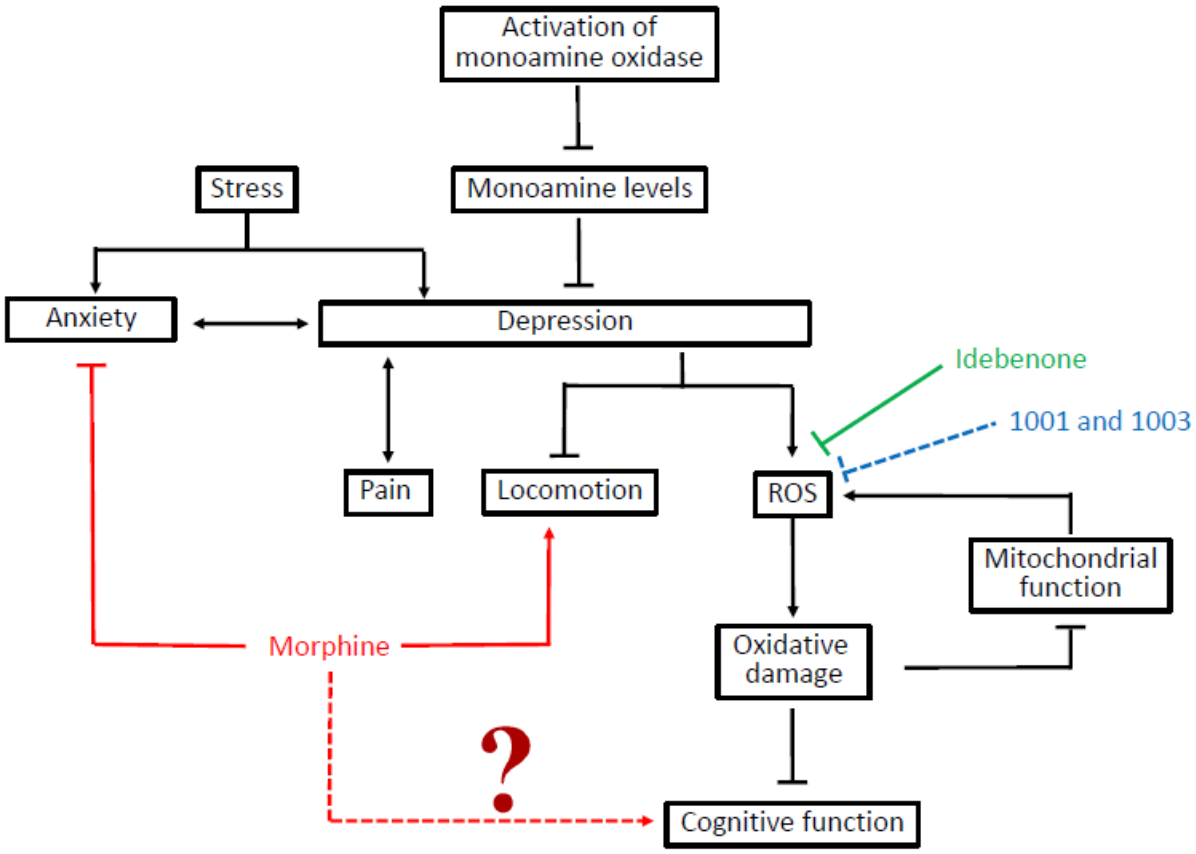

Figure 87. Possible mechanisms of the effects of opioids and idebenone on oxidative stress, depression and memory.
The levels of monoamines reduce followed by the activation of monoamine oxidase (MAO). Based on the monoamine theory, reduced monoamines causes depression. As consequences of depression, reduced locomotion and increased reactive oxygen species were observed. ROS further lead to damages and mitochondrial dysfunction, which return generated more ROS. In our study, oxidative stress damage resulted in memory loss in rats. This oxidative stress damage-induced memory loss can be reversed (in solid green line) or slightly reduced (in blue dash line) by antioxidant idebenone and novel bifunctional opioids (i.e., 1001 and 1003) respectively. On the other hand, exposure to stress contributes to the development of both depression and anxiety. In addition, anxiety and pain co-existence in depression. In our model, morphine treatment produced artificial antidepressant-like effect that were partially due to its effects on locomotion (in solid red line), anxiety (in solid red line) and cognitive function (in red dash line). However, the effect of morphine on memory function was not further confirmed in the novel object recognition model, a model of cognition (in red dash line).

9.5.3 Implications and future direction

The LH model as one of the frequently used models of depression was selected to investigate the antidepressant-like effects of both novel and conventional drugs in this study. In addition, in order to study depression-associated comorbidities, I developed a complex integrated animal

model based on the LH model. This is the first time such comprehensive model has been used to test the effects of drugs on different behaviours and the validity of this model was confirmed by including a known antidepressant (i.e., imipramine). Particularly, we evaluated different interference factors of the antidepressant-like effect of morphine in the LH model, including the number of avoidance events, mean escape latency, intertrial interval transfers and freezing. In this way, the results of this study are more accurate in terms of identifying artefacts and determining interfering factors, compared to previously published data (525,886). Therefore, my our results provide useful guidelines for future studies to modify and extend existing preclinical depression models and offers ideas on how to interpret data based on multiple behavioural paradigms.

In addition, our study sheds light on the puzzling relationships between oxidative stress, depression and depression-associated memory loss. In particular, it was surprising to observe that the cognitive effects of idebenone were restricted to recognition memory rather than the avoidance or escape learning. While the IS exposure *per se* impairs all three cognitive aspects in our model, which reflect both information encoding and recall, our idebenone data suggest that antioxidants may only be effective to normalize memory retrieval. To translate these results to clinical practice, based on our results we could conclude that trying to treat depression with antioxidants might not be a successful strategy. In contrast, a more promising approach would be to use antioxidants in patients with MDD to protect their cognitive function. This strategy might only be useful at a very early stage of cognitive dysfunction and might maintain their long-term memory or at least prevent further memory loss. It will be essential to test this hypothesis in careful controlled clinical trials to substantiate this claim. Given that idebenone has shown a very good safety profile in clinical trials (395), it offers itself as a promising starting point with minimal adverse effects for this type of therapeutic intervention in MDD

patients. In addition, this strategy could also be of great value under similar conditions where cognition is impaired due to elevated levels of stress such as in PTSD patients.

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Appendix

A1. Demonstration of missing contemporaneous controls for the 1001 and 1003 effects

Table 8. Mean ± S.E.M. of different controls that conducted in different calendar years and with different administration methods.

Conditions	Conducted in 2016, for idebenone experiment, oral administration					
	NS			IS		
Test sessions	EFs	S.E.M.	n	EFs	S.E.M.	n
T1	6	1.843909	5	23	2.67083	6
T2	3	1.048809	5	24.83333	1.720788	6
T3	8	3.507136	5	23.66667	1.256096	6
Conditions	Conducted in May 2015, for morphine experiment, s.c.					
	NS			IS		
Test sessions	EFs	S.E.M.	n	EFs	S.E.M.	n
T1	9.2	2.853069	5	25.875	1.125	6
T2	5.4	1.989975	5	24.25	1.600781	6
T3	5.4	3.264966	5	22.5	1.4516	6
Conditions	Conducted in 2015, for optimisation, no injection					
	NS			IS		
Test sessions	EFs	S.E.M.	n	EFs	S.E.M.	n
T1	6	1.073937	3	22.5	2.50383	3
T2	7	3.267427	3	23	5.794723	3
T3	6	2.193393	3	24.5	1.530273	3

Table 9. Multiple comparison between different controls.
No significance was found using repeated two-way ANOVA followed by Tukey comparison tests. (p<0.05 was considered as statistical significant)

Stat	Tukey's multiple comparisons test, repeated 2-way ANOVA	Mean Diff.	Significant	Adjusted P Value
NS groups				
T1	morphine, 2015 vs. opt., 2015	3.2	No	0.7688
	morphine, 2015 vs. idebenone, 2016	3.2	No	0.6335
	opt., 2016 vs. idebenone, 2016	0	No	> 0.9999
T2	morphine, 2015 vs. opt., 2015	-1.6	No	0.9359
	morphine, 2015 vs. idebenone, 2016	2.4	No	0.7719
	opt., 2016 vs. idebenone, 2016	4	No	0.6647
T3	morphine, 2015 vs. opt., 2015	-0.6	No	0.9907
	morphine, 2015 vs. idebenone, 2016	-2.6	No	0.7384
	opt., 2016 vs. idebenone, 2016	-2	No	0.9018
IS groups				
T1	morphine, 2015 vs. opt., 2015	3.375	No	0.5976
	morphine, 2015 vs. idebenone, 2016	2.875	No	0.4520
	opt., 2016 vs. idebenone, 2016	-0.5	No	0.9893
T2	morphine, 2015 vs. opt., 2015	1.25	No	0.9309
	morphine, 2015 vs. idebenone, 2016	-0.5833	No	0.9671
	opt., 2016 vs. idebenone, 2016	-1.833	No	0.8659
T3	morphine, 2015 vs. opt., 2015	-2	No	0.8331
	morphine, 2015 vs. idebenone, 2016	-1.167	No	0.8752
	opt., 2016 vs. idebenone, 2016	0.8333	No	0.9706

A2. Receptor activity of UTA1003

(The protocol and following unpublished results were provided by Alok Paul (PhD candidate, School of Medicine, University of Tasmania).

Opioid receptors belong to the inhibitory G_i linked G-protein coupled receptor subfamily (237). G proteins consist of three protein subunits, including α, β, γ subunits. In the inactive state the α, β and γ subunits form a heterotrimeric complex. In this complex, the α subunit binds guanosine diphosphate (GDP) (238). After opioid agonists bind to their specific opioid receptors, GDP is released from the α-subunit of G_i protein and was replaced by Guanosine-5'-triphosphate (GTP). The binding of GTP activates the α-subunit, leading to its disassociation from the βγ-complex. Subsequently, this G_i-α protein inhibits adenylyl cyclases, which further prevents the conversion of ATP to cAMP (239). Therefore, the activation of opioid receptors lead to a reduction of cellular cAMP. Based on this mechanism of using cAMP as a surrogate marker, the receptor activities of UTA1001 and UTA1003 were measured in Chinese Hamster Ovary (CHO) cell-lines expressing recombinant human opioid receptors (i.e., CHO-MOP (**Figure 88**) and CHO-DOP (**Figure 89**)). In addition, CHO wild type (CHO-WT) cells were used to confirm the receptor specificity of these two drugs (**Figure 87**). In this assay, CHO cells were incubated with 100 µM of UTA-opioids, the MOP agonist DAMGO or the DOP agonist DPDPE for 20 min in the presence of 1 µM of forskolin, which activates adenylyl cyclase and increases intracellular levels of cAMP. Control cells were only incubated with 1 µM of forskolin for same time period. Data was represented as % inhibition of forskolin-induced cAMP production.

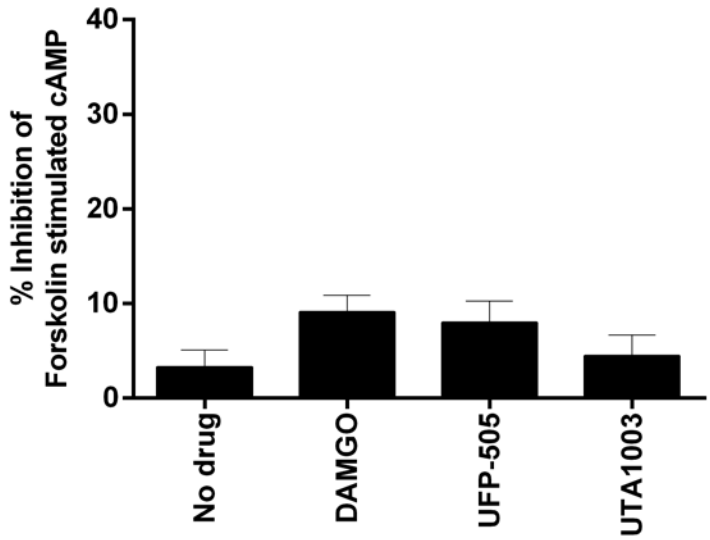

Figure 88. Opioid receptor specificity assessment of UTA-opioids using wild-type cells.
No significant differences were detected in all treated groups, compared to the non-treated cells.

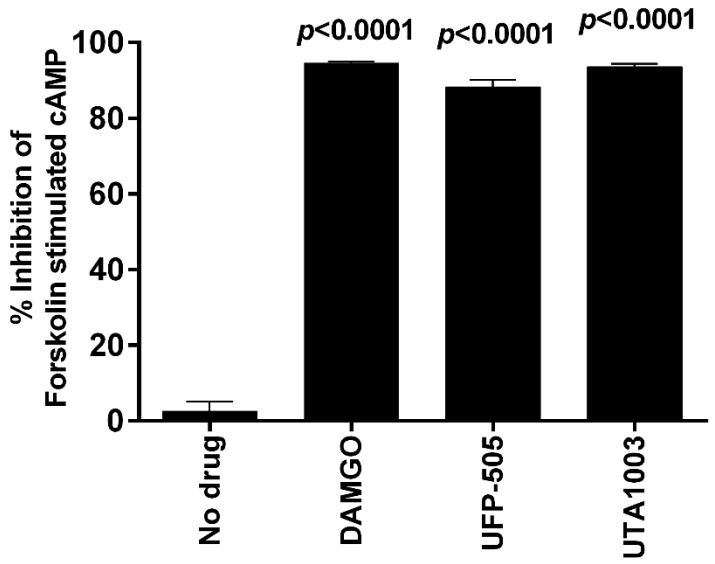

Figure 89. Effects of UTA-opioids and DAMGO on μ-opioid (MOP) receptor expressing cells.
Statistically significant differences ($p<0.0001$) against the effect of 1 μM forskolin (no drug) were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6).

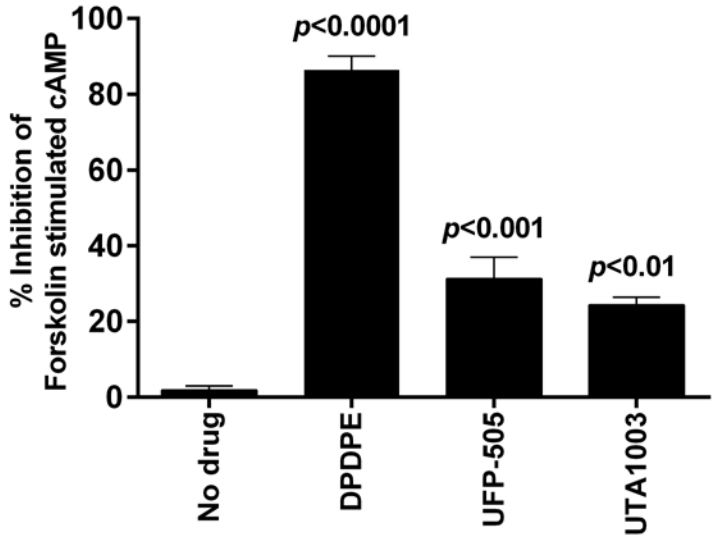

Figure 90. Effects of UTA-opioids and DPDPE on δ-opioid (DOP) receptor expressing cells.
Statistically significant differences against the effect of 1 μM forskolin (no drug) were calculated using one-way ANOVA with Dunnett's multiple comparisons test. Values represent the mean ± SEM (n = 6).

A3. Antinociceptive effects of 1001 and 1003 in vivo

The testing protocol and the unpublished results of the tail flick (TF) assay to measure antinociception were provided by Alok Paul (PhD candidate, School of Medicine, University of Tasmania).

Baseline antinociception of male SD rats (8 weeks old) was measured before drug administration. Equimolar concentrations of UTA1001 (27.1 mg/kg) and UTA1003 (24.6 mg/kg) were injected once and their antinociceptive effects were evaluated in the TF assay 15, 30, 60 and 120 min after injection. MPE (maximum possible effect, defined as MPE % or antinociception = 100 × [(test latency – baseline latency) / (cut-off time (15 s) – baseline latency)]) was used to simplify the comparison between different drugs at different time points against the basal level.

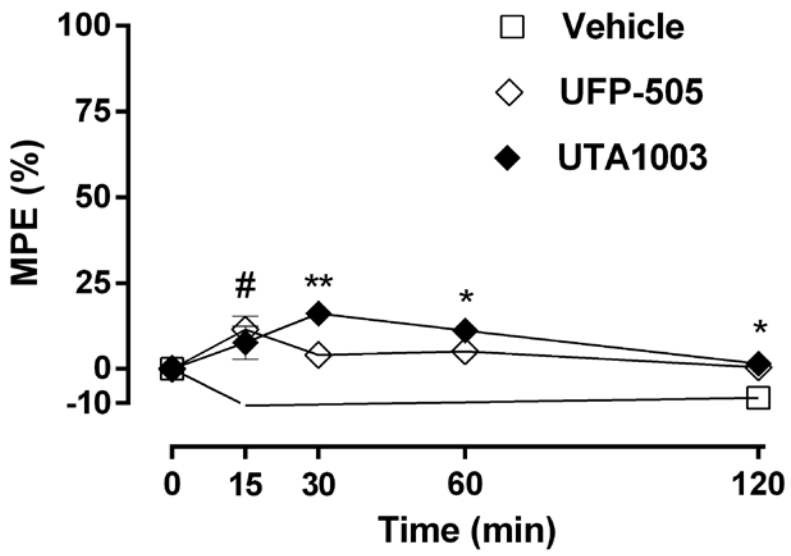

Figure 91. Antinociceptive effects of UTA1001 (UFP-505) and 1003.
Statistical significance against the effect of vehicle at the same testing point was calculated using student's t-test and is shown as $*p<0.05$, $**p<0.01$ in the UTA1003-treated group and $^{\#}p<0.01$ in the UTA1001 (UFP-505)-treated group. Values represent mean ± SEM (n = 6 animals per group).

A4. Animal batches of the morphine-imipramine experiment and the allocations of animals

Table 10. Animal numbers in weekly batches and excluded rats in each group.

	Batch	Animal numbers
Delivery	week 1	180, 181,183, 185
	week 2	213, 214, 215, 216
	week 3	242, 243, 244, 245
	week 4	252, 253, 254
	week 5	276, 277, 278, 280
	week 6	336, 337, 338, 339
	week 7	367, 368, 369
	week 8	402, 403, 404
	week 9	453, 454, 455, 456
	week 10	468, 469, 470, 471
	week 11	512, 513, 514
Excluded rats	NS	SD 242, 243, 256
	IS	SD 253, 278
	ES	SD 216, 454, 513
	MOR	SD 455
	IMI	SD 339, 404

Table 11. Rat numbers and body weights in each group
(Colour coding is the same as that in the Table 10, aiming to exhibit the weekly rat allocations from week 1 to 5)

	Experimental Period				
Rat IDs	**D1**	**D2**	**D4**	**D5**	**D6**
IS group					
SD180	258	248	246	254	256
SD185	264	256	256	262	264
SD214	304	296	290	296	286
SD244	278	278	280	280	284
SD245	284	286	286	286	288
SD254	306	306	306	306	308
NS group					
SD183	264	252	248	252	268
SD215	326	320	312	312	318
SD252	356	354	346	356	348
SD276	346	348	342	342	350
SD277	304	306	312	308	312
IS+MOR group					
SD336	206	198	192	200	204
SD367	204	198	198	204	206
SD368	230	226	222	230	232
SD402	210	204	204	212	220
SD403	178	176	176	182	184
SD453	246	246	242	248	250
SD468	216	210	200	202	210
IS+IMI group					
SD369	204	204	188	204	204
SD456	258	254	244	248	250
SD469	214	206	196	198	202
SD470	212	206	198	200	200
SD471	218	216	184	208	210
SD512	218	220	204	208	208
ES group					
SD181	264	258	256	260	268
SD213	302	290	286	291	298
SD280	368	358	354	360	362
SD337	204	204	201	201	205
SD338	210	208	204	207	210
SD514	214	214	208	210	214

A5. Animal Ethics Approval Permit

	University of Tasmania Animal Ethics Committee **ETHICS APPROVAL PERMIT**	University of Tasmania Office of Research Services Ph: 03 62267283 Fax: 03 62267148 animal.ethics@utas.edu.au

To:	Dr Nikolaos Dietis
From:	Marilyn Pugsley Executive Officer Animal Ethics
Date:	2 June 2014
Project:	A13857 – The effects of novel bifunctional opioids in a rat-model of depression
Approved on:	2 June 2014
Approval expires:	2 June 2017
1st Annual Report due **before**:	**2 June 2015**

Please read this permit carefully as **approval may be withdrawn** for projects that do not comply with the conditions

The Animal Ethics Committee has approved the above project and a copy of the document is attached. The approval is subject to the review and approval of an annual report which is due before the approval anniversary**. Please note this date in your diary.**

This approval constitutes ethical clearance by the Animal Ethics Committee. If this project involves the conduct of a Veterinary Service or Other Animal Service as defined in the Veterinary Surgeons Act 1987 (Tas) and Veterinary Surgeons Regulations 2012 (Tas), it is your responsibility to ensure that the project is conducted in accordance with the provisions of the Act and Regulations. Please contact the Animal Welfare Officer, Dr Sue Ottomanski (6226 7491 or sue.ottomanski@utas.edu.au) to discuss veterinary procedure competency accreditation.

As the Responsible Investigator, you MUST ensure that:

(a) all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

(b) a full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

(c) you contact the Animal Welfare Officer, Dr Sue Ottomanski (sue.ottomanski@utas.edu.au) to advise her when and where your experiments will be conducted. Sufficient notice needs to be given so that if the AWO wishes to make an inspection, this can be easily arranged.

(d) That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The Animal Ethics Committee is to be promptly notified of any unexpected events which occur during the period of the approved project and impact on the welfare of the animals.

Autopsy should be performed by a qualified veterinarian when animals die unexpectedly. Any foreseeable departure from this requirement must have been outlined and approved in the initial application.

If the project is to continue past the expiry date, a new initial application will need to be submitted. A project can only be approved for a maximum of 3 years.

If the investigation necessitates a Parks & Wildlife permit you are required to send a copy of this permit to the AEC Secretary before commencing work.

Marilyn Pugsley
Executive Officer Animal Ethics

	University of Tasmania **Animal Ethics Committee**
Ethics Number	A13857
Project Name	The effects of novel bifunctional opioids in a rat-model of depression
Chief Investigator	Dr Nikolaos Dietis
School	Pharmacy
Person responsible for day-to-day care	
Ethics start date	2 June 2014
Ethics approved to	2 June 2017 (with annual renewal)
Emergency Contact	